证 明

CERTIFICATE

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国 际 申 请 号: PCT/CN02/00635
INTERNATIONAL APPLICATION NUMBER

国 际 申 请 日: 09 SEP 2002(09.09.02)
INTERNATILNAL FILING DATE

发 明 名 称: PHARMACEUTICAL COMPOSITION AND METHOD OF TREATMENT OF HUMAN MALIGNANICES WITH ARGININE DEPRIVATION
TITLE OF INVENTION

申 请 人: BIO—CANCER TREATMENT INTERNATIONAL LIMITED
APPLICANT

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

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王景川

二零零三年九月二十六日
SEPTEMBER 26, 2003

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PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No. PCT/CN 02/ 0 0 6 3 5

International Filing Date 09 SEP 2002(09.09.02)

RO/CN 中华人民共和国国家知识产权局 PCT International Application

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* P2002221C

Box No. I TITLE OF INVENTION

PHARMACEUTICAL COMPOSITION AND METHOD OF TREATMENT OF HUMAN MALIGNANICES WITH ARGININE DEPRIVATION

Box No. II APPLICANT ☐ This person is also inventor

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

BIO-CANCER TREATMENT INTERNATIONAL LIMITED

Room 1619, 16/F., Central Building, 1 Pedder Street, Central, Hong Kong, CN

Telephone No.

Facsimile No.

Teleprinter No.

Applicant's registration No. with the Office

State *(that is, country)* of nationality: CN

State *(that is, country)* of residence: CN

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

CHENG, Ning~~[Man]~~ man

Flat 6B, Block 1, Clovelly Court, 12 May Road, Hong Kong, CN

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

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State *(that is, country)* of residence: CN

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

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Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

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NTD Patent & Trademark Agency Ltd.

10th Floor, Block A, Investment Plaza, 27 Jinrongdajie, Beijing 100032, P.R. China

Telephone No. 86-10-66211836

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Teleprinter No.

Agent's registration No. with the Office

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

RO/CN

Sheet No. ...2...

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

LEUNG, Yun ~~Chung~~ chung

Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Kowloon, Hung Hom, Hong Kong, CN

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office:

State *(that is, country)* of nationality: CN

State *(that is, country)* of residence: CN

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

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State *(that is, country)* of nationality: CN

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This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

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This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office:

State *(that is, country)* of nationality:

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This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office:

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This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

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Box No. V DESIGNATION OF STATES *Mark the applicable check-boxes below; at least one must be marked.*

The following designations are hereby made under Rule 4.9(a):

Regional Patent

☒ AP **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, MZ Mozambique, SD Sudan, SL Sierra Leone, SZ Swaziland, TZ United Republic of Tanzania, UG Uganda, ZM Zambia, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

☒ EA **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT

☒ EP **European Patent:** AT Austria, BE Belgium, BG Bulgaria, CH & LI Switzerland and Liechtenstein, CY Cyprus, CZ Czech Republic, DE Germany, DK Denmark, EE Estonia, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, SK Slovakia, TR Turkey, and any other State which is a Contracting State of the European Patent Convention and of the PCT

☒ OA **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GQ Equatorial Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

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Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM

The priority of the following earlier application(s) is hereby claimed:

Filing date of earlier application *(day/month/year)*	Number of earlier application	Where earlier application is:		
		national application: country or Member of WTO	regional application:* regional Office	international application: receiving Office
item (1) June 20, 2002 (20/06/2002)	60/390,757	USA		
item (2)				
item (3)				
item (4)				
item (5)				

☐ Further priority claims are indicated in the Supplemental Box.

The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of this international application is the receiving Office)* identified above as:

☐ all items ☐ item (1) ☐ item (2) ☐ item (3) ☐ item (4) ☐ item (5) ☐ other, see Supplemental Box

** Where the earlier application is an ARIPO application, indicate at least one country party to the Paris Convention for the Protection of Industrial Property or one Member of the World Trade Organization for which that earlier application was filed (Rule 4.10(b)(ii)):* ....

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)*:

ISA / CN

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority)*:

Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII DECLARATIONS

The following **declarations** are contained in Boxes Nos. VIII (i) to (v) *(mark the applicable check-boxes below and indicate in the right column the number of each type of declaration)*: Number of declarations

☐ Box No. VIII (i)	Declaration as to the identity of the inventor	:
☐ Box No. VIII (ii)	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	:
☐ Box No. VIII (iii)	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	:
☐ Box No. VIII (iv)	Declaration of inventorship (only for the purposes of the designation of the United States of America)	:
☐ Box No. VIII (v)	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	:

Sheet No. 5

Box No. IX CHECK LIST; LANGUAGE OF FILING

This international application contains:

(a) the following number of sheets in paper form:

request (including declaration sheets)	:	5
description (excluding sequence listing part)	:	46
claims	:	4
abstract	:	1
drawings	:	38
Sub-total number of sheets	:	94
sequence listing part of description *(actual number of sheets if filed in paper form, whether or not also filed in computer readable form; see (b) below)*	:	1
Total number of sheets	:	95

(b) sequence listing part of description filed in **computer readable form**

(i) ☐ only (under Section 801(a)(i))

(ii) ☐ in addition to being filed in paper form (under Section 801(a)(ii))

Type and number of carriers (diskette, CD-ROM, CD-R or other) on which the sequence listing part is contained *(additional copies to be indicated under item 9(ii), in right column)*:

.....

This international application is accompanied by the following item(s) *(mark the applicable check-boxes below and indicate in right column the number of each item)*:

		Number of items
1. ☒ fee calculation sheet	:	1
2. ☐ original separate power of attorney	:	
3. ☐ original general power of attorney	:	
4. ☐ copy of general power of attorney; reference number, if any:	:	
5. ☐ statement explaining lack of signature	:	
6. ☐ priority document(s) identified in Box No. VI as item(s):	:	
7. ☐ translation of international application into *(language)*:	:	
8. ☐ separate indications concerning deposited microorganism or other biological material	:	
9. ☐ sequence listing in computer readable form (indicate also type and number of carriers (diskette, CD-ROM, CD-R or other))		
(i) ☐ copy submitted for the purposes of international search under Rule 13*ter* only (and not as part of the international application)	:	
(ii) ☐ *(only where check-box (b)(i) or (b)(ii) is marked in left column)* additional copies including, where applicable, the copy for the purposes of international search under Rule 13*ter*	:	
(iii) ☐ together with relevant statement as to the identity of the copy or copies with the sequence listing part mentioned in left column	:	
10. ☐ other *(specify)*:	:	

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: English

Box No. X SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

NTO PATENT & TRADEMARK AGENCY LIMITED * HONG KONG *

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1. Date of actual receipt of the purported international application: 09 SEP 2002 (09.09.02)

2. Drawings: ☐ received: ☐ not received:

3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:

4. Date of timely receipt of the required corrections under PCT Article 11(2):

5. International Searching Authority (if two or more are competent): ISA /

6. ☐ Transmittal of search copy delayed until search fee is paid

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Date of receipt of the record copy by the International Bureau:

This sheet is not part of and does not count as a sheet of the international application.

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FEE CALCULATION SHEET
Annex to the Request

For receiving Office use only

International Application No. PCT/CN 02/00635

09 SEP 2002 (09.09.02)
Date stamp of the receiving Office

Applicant's or agent's file reference: P2002221C

Applicant
BIO-CANCER TREATMENT INTERNATIONAL LIMITED

CALCULATION OF PRESCRIBED FEES

			Receiving Office use
1. TRANSMITTAL FEE	CNY500	T	CNY500.-
2. SEARCH FEE	CNY1500	S	CNY1500.-

International search to be carried out by CN
(If two or more International Searching Authorities are competent to carry out the international search, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE
Basic Fee

Where item (b) of Box No. IX applies, enter **Sub-total number of sheets**
Where item (b) of Box No. IX does not apply, enter **Total number of sheets** } 95

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b1	first 30 sheets	CHF650	b1	CHF650.-
b2	65 (number of sheets in excess of 30) x 15 (fee per sheet) =	CHF975	b2	CHF975.-
b3	additional component (only if sequence listing part of description is filed in computer readable form under Section 801(a)(i), or both in that form and on paper, under Section 801(a)(ii)): 400 x ____ (fee per sheet) =		b3	
	Add amounts entered at b1, b2 and b3 and enter total at B	CHF1625	B	CHF1625.-

Designation Fees
The international application contains 89 designations.

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5 (number of designation fees payable *(maximum 5)*) x 140 (amount of designation fee) =	CHF700	D	CHF700.-
Add amounts entered at B and D and enter total at I	CHF2325	I	CHF2325.-

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

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4. FEE FOR PRIORITY DOCUMENT *(if applicable)*		P	
5. TOTAL FEES PAYABLE — Add amounts entered at T, S, I and P, and enter total in the TOTAL box	CHF2325, CNY2000	TOTAL	CHF2325.- CNY2000.-

☐ The designation fees are not paid at this time.

MODE OF PAYMENT

☒ authorization to charge deposit account (see below)
☐ postal money order
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☐ other *(specify)*:

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☐ *(This check-box may be marked only if the conditions for deposit accounts of the receiving Office so permit)* Authorization to charge any deficiency or credit any overpayment in the total fees indicated above.

☐ Authorization to charge the fee for priority document.

Receiving Office: RO/ CN
Deposit Account No.:
Date: September 9, 2002
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NTD PATENT & TRADEMARK AGENCY LIMITED HONG KONG 中国国际贸易促进委员会专利商标事务所 专用章

See Notes to the fee calculation sheet

PHARMACEUTICAL COMPOSITION AND METHOD OF TREATMENT OF HUMAN MALIGNANCIES WITH ARGININE DEPRIVATION

FIELD OF INVENTION

[0001] The present invention is related to pharmaceutical compositions and use therefor. In particular, the present invention is related to pharmaceutical compositions that have the capability of reducing the arginine level in patients with tumours and its use for treatment of human malignancies.

BACKGROUND OF INVENTION

[0002] Arginase I (EC 3.5.3.1; L-arginine amidinohydrolase), is a key mammalian liver enzyme that catalyses the final step in the urea formation in the Urea cycle, converting arginine into ornithine and urea. Rat liver extract, which has a high content of arginase, was found to have anti-tumour properties in vitro when it was accidentally added to tumour cell culture medium (Burton et al., 1967 Cytolytic action of corticosteroids on thymus and lymphoma cells in virto. Can J. Biochem. 1967 Feb; 45(2):289-97). Subsequent experiments showed that the anti-tumour properties of the enzyme were due to depletion of arginine, which is an essential amino acid in the culture medium. At below 8 μM levels of arginine, irreparable cell death in cancer cells occurred (Storr & Burton, (1974). The effects of arginine deficiency on lymphoma cells. Br. J. Cancer 30, 50).

[0003] A more novel aspect of arginine centers on its role as the direct precursor for the synthesis of the potent signalling molecule nitric oxide (NO), which functions as a neurotransmitter, smooth muscle relaxant, and vasodilator. Biosynthesis of NO involves a Ca^{++}, NADPH-dependent reaction catalysed by

nitric oxide synthase. Another recognized role of arginine is that it acts as a precursor, via ornithine, of the polyamines, spermidine and spermine, which participate in diverse physiologic processes including cell proliferation and growth.

[0004] Arginine also serves as a substrate for several important enzymes, including nitric oxide synthase (NOS). There are three types of NOSs, nNOS, eNOS and iNOS, all convert arginine to nitric oxide and citrulline. The facial flushes induced by NO, for instance, is mediated through nNOS, the neuronal type of NOS. iNOS, the inducible NOS is produced by macrophages and the NO so produced from arginine during septicaemia causes vasodilation in endotoxic shock. eNOS, the endothelial NOS, is produced by endothelial cells in blood vessels. It converts arginine into NO, which then causes de-aggregation of platelets in the endothelial surfaces through cGMP mechanism. NO produced from eNOS in the local endothelial lining has a half-life of about 5 seconds and diffusion distance of about 2 microns.

[0005] The productions of these enzymes are controlled by different NOS genes (NOS1, NOS2, NOS3) encoded in chromosomes 12, 17 & 7, respectively. These genes share strikingly similar genomic structures in size of exons and the location of the splice junctions.

[0006] The *in vitro* anti-tumour activities of arginine depletion were confirmed recently by a group in Scotland, UK (Scott et al., 2000 Single amino acid (arginine) deprivation: rapid and selective death of cultured transformed and malignanat cells. Br. J. Cancer 83, 800-810; Wheatley et al. 2000 Single amino acid (arginine) restriction: Growth and Death of cultured HeLa and Human Diploid Fibroblasts. Cellular Physiol. Biochem. 10, 37-55). Of the 24 different tumour cell lines tested, which included common cancers such as breast, colorectal, lung, prostate and

ovaries, all died within 5 days of arginine depletion. Using flow-cytometry studies, the group was able to show that normal cell lines would enter into quiescence for up to several weeks in G0 phase of the cell cycle without any apparent harm. Tumour cells, however, would proceed pass the "R" point in the G1 phase and enter the S phase with deficiency of arginine. Without arginine, which is an irreplaceable amino acid, protein synthesis is deranged. Some cell lines were shown to die from apoptosis. More excitingly, repeated depletions can bringforth tumour kill without "resistant" being developed (Lamb et al. 2000 Single amino acid (arginine) deprivation induces G1 arrest associated with inhibition of Cdk4 expression in cultured human diploid fibroblasts. Experimental Cell Research 225, 238-249).

[0007] Despite the promising *in vitro* data, attempts with arginine depletion to treat cancer *in vivo* were unsuccessful. The original Storr group attempted to treat tumour- bearing rats with intraperitoneal liver extracts and met with no success. (Storr et al. Br. J. Cancer, 30: 50, 1974) It is now generally recognized that under normal physiological condition, the plasma arginine level and indeed that of other amino acids too, are kept between the normal ranges (100-120 μM) with muscle being the main regulator. In the face of amino acid deficiency, intracellular protein breakdown pathways are activated (proteasomal and lysosomal) releasing amino acids into the circulation. This amino acid homeostatic mechanism keeps the various amino acid levels at constant ranges. Thus, previous attempts to deplete arginine with various physical methods or arginine degrading enzymes have failed because of the body' s amino acid homeostatic mechanism.

[0008] To overcome the problem on the body' s natural homeostatic tendencies, Tepic et al. in US Patent 6,261,557 described a therapeutic composition and

method for treatment of cancer in which an arginine decomposing enzyme is used in combination with a protein breakdown inhibitors such as insulin in order to prevent the muscles of the body from replenishing the depleted arginine.

[0009] Although insulin can act as a protein breakdown inhibitor, it also has far-reaching physiological effects on the human body that may cause fatal problems if blood glucose levels of the patient are not strictly maintained within the narrow normal range. **It is therefore an object to the present invention to find improved method of treatment and compositions for the treatment of cancer.**

SUMMARY OF INVENTION

[0010] Accordingly, the present invention provides a method to produce a modified relative stable human arginase, in particular, human arginase I, by recombinant cloning and expression of such in a selected bacterial strain, prefer *Bacillus subtilis*. Furthermore, the present invention provides pharmaceutical compositions comprising modified human arginase, and uses it, for treatment of disease without further in combination with a protein breakdown inhibitor.

[0011] In accordance with the present invention, there is provided an isolated and substantially purified recombinant human arginase that has sufficiently high enzymatic activity and stability to maintain "adequate arginine deprivation" (hereinafter referred to as "AAD") in a patient for at least 3 days.

[0012] As used herein, the term "isolated" refers to a form that does not occur in nature. The term "substantially purified" refers to at least about 95%, preferably at least about 99%, free of other components used to produce and/or modify the protein.

[0013] As used herein, the phrase "substantially the same," whether used in reference to the nucleotide sequence of DNA, the ribonucleotide sequence of RNA, or the

amino acid sequence of protein, refers to sequences that have slight and non-consequential sequence variations from the actual sequences disclosed herein. Species that are substantially the same are considered to be equivalent to the disclosed sequences and as such are within the scope of the appended claims. In this regard, "slight and non-consequential sequence variations" means that sequences that are substantially the same as the DNA, RNA, or proteins disclosed and/or claimed herein are functionally equivalent to the sequences disclosed and/or claimed herein. Functionally equivalent sequences will function in substantially the same manner to produce substantially the same compositions as the nucleic acid and amino acid compositions disclosed and claimed herein. In particular, functionally equivalent DNAs encode proteins that are the same as those disclosed herein or proteins that have conservative amino acid variations, such as substitution of a non-polar residue for another non-polar residue or a charged residue for a similarly charged residue. These changes include those recognized by those of skill in the art not to substantially alter the tertiary structure of the protein.

[0014] As used herein, the term "sufficiently high enzymatic activity" refers to the enzyme specific activity of the recombinant human arginase for at least about 250 U/mg, preferably at least about 280 U/mg, more preferably at least about 300 U/mg, and the most preferably at least about 330 U/mg. In the preferred embodiment, the isolated and purified recombinant human arginase has a specific activity of about 330 U/mg.

[0015] As used herein, the term "stability" refers to *in vitro* (as described in Example 9B) and *in vivo* (as described in Example 9B) stability of the recombinant human arginase. More preferably, the stability refers to *in vivo* stability. The rate of decrease of enzyme activity is inversely proportional to the serum/plasma

stability of the isolated, purified recombinant human arginase. The half-life of such a human arginase in serum/plasma is calculated according to formulae known in the art.

[0016] As used herein, the term "adequate arginine deprivation" (AAD) refers to *in vivo* arginine level at or below 10μM.

[0017] As used herein, the term "half time" (1/2-life) refers to the time that would be required for the concentration of the isolated and substantially purified recombinant human arginase in serum/plasma or other body fluid, either *in vivo* or *in vitro*, to fall by half.

[0018] In the preferred embodiment, the isolated and substantially purified recombinant human arginase enzyme is expressed in *B. subtilis* and modified by pegylation to improve stability and minimise immunoreactivity. The pegylated isolated and purified recombinant human arginase has a serum/plasma ½-life of about at least 3 days and specific activity of about at least 255 U/mg.

[0019] In accordance with the present invention, there are provided pharmaceutical compositions comprising the isolated and substantially purified recombinant human arginase having sufficiently high enzymatic activity and stability to maintain AAD in a patient for at least 3 days. Such recombinant human arginase is further modified by pegylation to improve stability and minimise immunoreactivity as described above.

[0020] **According to another aspect of the present invention, the invention pharmaceutical composition is further formulated.**

[0021] Formulations of the pharmaceutical composition of the present invention can be used in the form of a solid, a solution, an emulsion, a dispersion, a micelle, a liposome, and the like, wherein the resulting formulation contains one or more of the

modified human arginase in the practice of the present invention, as active ingredients, in admixture with an organic or inorganic carrier or excipient suitable for enteral or parenteral applications. The active ingredients may be the isolated and substantially purified recombinant human arginase, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, and any other form suitable for use in manufacturing preparations, in solid, semisolid, or liquid form. In addition auxiliary, stabilizing, thickening and coloring agents and perfumes may be used. The active ingredients of one or more isolated and substantially purified recombinant human arginase are included in the pharmaceutical formulation in an amount sufficient to produce the desired effect upon the target process, condition or disease.

[0022] Pharmaceutical formulations containing the active ingredients contemplated herein may be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsions, hard or soft capsules, or syrups or elixirs. Formulations intended for oral use may be prepared according to any method known in the art for the manufacture of pharmaceutical formulations. The tablets may be uncoated or they may be coated by known techniques to delay disintegration and absorption in the gastrointestinal tract, thereby providing sustained action over a longer period. They may also be coated to form osmotic therapeutic tablets for controlled release.

[0023] In some cases, formulations for oral use may be in the form of hard gelatin capsules wherein the active ingredients are mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate, kaolin, or the like. They may also be in the form of soft gelatin capsules wherein the active ingredients are mixed with water or an oil medium, for example, peanut oil, liquid paraffin, or olive oil.

[0024] The pharmaceutical formulations may also be in the form of a sterile injectable solution or suspension. This suspension may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,4-butanediol. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides, fatty acids (including oleic acid), naturally occurring vegetable oils like sesame oil, coconut oil, peanut oil, cottonseed oil, or synthetic fatty vehicles, like ethyl oleate, or the like. Buffers, dextrose solutions preservatives, antioxidants, and the like, can be incorporated or used as solute to dissolve the soluble enzyme as required.

[0025] In accordance with the present invention, a method for treatment of a disease is provided for administering a formulated pharmaceutical composition of the present invention to a patient to maintain the arginine level in such a patient to below 10 μM for at least 3 days without the need for other protein breakdown inhibitors. In one of the preferred embodiments, no insulin is administered exogenously for non-diabetic patients. As used herein, the term "disease" refers to any pathological conditions, including but not limited to liver diseases and cancer.

[0026] Furthermore, the treatment method of the present invention involves the monitoring of the patient's blood for platelet count (preferably maintained above 50,000 x 10^9) and prothrombin time (maintained at less than 4 times normal). No nitric oxide producer is exogenous administered unless these levels of platelet count and prothrombin time are reached.

[0027] In the preferred embodiment of the present invention, pegylated modification of the isolated and substantially purified recombinant human arginase is given as short infusion of over 1 hour at 1 mg/kg in 2 doses 12 hours apart. On day 2, arginase infusion is given at 2 mg/kg /day as 24 hours infusion. Arginine levels and arginase activity are taken twice daily before arginase infusion. If AAD is not achieved, the dose of the next infusion of arginase is increased by 20% until AAD is achieved. The maximum tolerated duration of AAD is defined as the period of time during which blood pressure is under control (with or without medication as deem appropriate by the treating physician) platelet count above 50,000 $x10^9$ and prothrombin time less than 4x normal. As with arginine levels, complete blood count (CBC) and prothrombin time (PT) are taken twice per day. Liver chemistry is monitored at least on alternate days during the treatment.

[0028] In a further embodiment of the present invention, the pegylated modification of the isolated and substantially purified recombinant human arginase is administered to augment the endogenous arginase released after liver embolization with lipiodol and gel foam.

[0029] Therefore, the present invention provides a pegylated modification of an isolated and substantially purified recombinant human_arginase produced by cloning and expression such an enzyme using *B. subtilis*, a pharmaceutical composition comprising same, and a method of use therefor. The invention enzyme and its pharmaceutical composition is sufficiently stable with an appropriate serum/plasma ½-life to be advantageously administered in short infusions so that it is possible for the treatment course to be provided out-patient or in a clinic to reduce costs. Furthermore, the invention enzyme and its pharmaceutical

composition has sufficiently high activity to be used as a single drug in the absence of an exogenously administered protein degradation enzyme such as insulin.

BRIEF DESCRIPTION OF DRAWINGS

[0030] Figure 1 shows plasmid map of pAB101. This plasmid carries the gene encoding arginase (arg) and only replicates in *E. coli* but not in *B. subtilis*.

[0031] Figures **2A and 2B** shows nucleotide sequence and its deduced amino acid sequence of the human arginase.

[0032] Figure 2A shows the nucleotide sequence (SEQ ID NO: 1) from EcoRI/MunI to XbaI sites of plasmid pAB101. Nucleotide (nt) 1-6, EcoRI/MunI site; nt 481-486, -35 region of promoter 1; nt 504-509, -10 region of promoter 1; nt 544-549, -35 region of promoter 2; nt 566-571, -10 region of promoter 2; nt 600-605, ribosome binding site; nt 614-616, start codon; nt 632-637, NdeI site; nt 1601-1603, stop codon; nt 1997-2002, XbaI site.

[0033] Figure 2B shows the encoding nucleotide sequence (SEQ ID NO: 2) and its corresponding encoded amino acid sequence (SEQ ID NO: 3) of the human arginase. Nucleotide 614-1603 from Figure 2A is a encoding region for the amino acid sequence of arginase. The 6xHis (SEQ ID NO: 4) tag at the N-terminus is underlined. Translation stop codon is indicated by asterisk.

[0034] Figure 3 is a schematic drawing of the construction of a *B. subtilis* prophage allowing expression of arginase.

[0035] Figure 4 shows the time-course for fermentation in a 2-liter fermentor by the recombinant *Bacillus subtilis* prophage strain. Figure 4A shows the results obtained from the batch fermentation. Figure 4B shows the results obtained from the fed-batch fermentation.

[0036] Figure 5 shows history plots of the fermentation showing the changes of parameters such as temperature, stirring speed, pH and dissolved oxygen values. Figure 5A shows the history plot from the batch fermentation. Figure 5B shows the history plot from the fed-batch fermentation.

[0037] Figures 6A and 6B show the results of biochemical purification of human arginase at 3 h after heat shock by the first 5-ml HiTrap Chelating column. Figure 6A shows the FPLC running parameters and protein elution profile. Figure 6B shows the SDS-PAGE (12 %) analysis of 5 μl of each of the fractions 11-31 collected from the column. The protein gel was stained with coomassie brilliant blue and destained to show the protein bands. Lane M: low-range molecular weight marker (1 μg per band; Bio-Rad), with MW (Daltons): 97,400; 66,200; 45,000; 31,000; 21,500; 14,400.

[0038] Figures 7A and 7B show results of purification of the human arginase at 3 h after heat shock by the second 5-ml HiTrap Chelating column. Figure 7A shows the FPLC running parameters and protein elution profile. Figure 7B shows the SDS-PAGE (12 %) analysis of 1 μl of each of the fractions 9-39 collected from the column. The protein gel was stained with coomassie brilliant blue and destained to show the protein bands. Lane M: low-range molecular weight marker (1 μg per band; Bio-Rad), with MW (Daltons): 97,400; 66,200; 45,000; 31,000; 21,500; 14,400.

[0039] Figures 8A and 8B show results of purification of the human arginase at 6 h after heat shock by the first 5-ml HiTrap Chelating column. Figure 8A shows FPLC running parameters and protein elution profile. Figure 8B shows the SDS-PAGE (12 %) analysis of 2.5 μl of each of the fractions 10-32 collected from the

column. The protein gel was stained with coomassie brilliant blue and destained to show the protein bands. Lane M: low-range molecular weight marker (1 μg per band; Bio-Rad), with MW (Daltons): 97,400; 66,200; 45,000; 31,000; 21,500; 14,400.

[0040] Figures 9A and 9B show results of purification of the human arginase at 6 h after heat shock by the second 5-ml HiTrap Chelating column. Figure 9A shows FPLC running parameters and protein elution profile. Figure 9B shows the SDS-PAGE (12 %) analysis of 2 μl of each of the fractions 8-E6 collected from the column. The protein gel was stained with coomassie brilliant blue and destained to show the protein bands. Lane M: low-range molecular weight marker (1 μg per band; Bio-Rad), with MW (Daltons): 97,400; 66,200; 45,000; 31,000; 21,500; 14,40.

[0041] Figure 10 shows the time-course of bacterial cell growth when heat shock was performed at a higher cell density. Heat shock was performed at 8 h when the culture density (OD_{600nm}) was about 25.

[0042] Figure 11 is the history plot of the fed-batch fermentation when heat shock was performed at a higher cell density. This plot shows the changes of parameters such as temperature, stirring speed, pH and dissolved oxygen values.

[0043] Figures 12A and 12B show the results of purification of the human arginase at 6 h after heat shock (at a higher cell density of OD 25) by the first 5-ml HiTrap Chelating column. Figure 12A shows FPLC running parameters and protein elution profile. Figure 12B shows results of SDS-PAGE (12 %) analysis of 5 μl of each of the fractions 16-45 collected from the column. The protein gel was stained

with coomassie brilliant blue and destained to show the protein bands. Lane M: low-range molecular weight marker (1 μg per band; Bio-Rad), with MW (Daltons): 97,400; 66,200; 45,000; 31,000; 21,500; 14,400. Lane "crude" : 5 μl of the crude cell extract before loading the column.

[0044] Figures 13A and 13B show the results of purification of the human arginase at 6 h after heat shock (at a higher cell density of OD 25) by the second 5-ml HiTrap Chelating column. Figure 13A shows FPLC running parameters and protein elution profile. Figure 13B shows the SDS-PAGE (12 %) analysis of 5 μl of each of the fractions 7-34 collected from the column. The protein gel was stained with coomassie brilliant blue and destained to show the protein bands. Lane M: low-range molecular weight marker (1 μg per band; Bio-Rad), with MW (Daltons): 97,400; 66,200; 45,000; 31,000; 21,500; 14,400.

[0045] Figure 14 shows the results of purification of the human arginase at 6 h after heat shock (at a higher cell density of OD 25) by the first 1-ml HiTrap SP FF column. Figure 14A shows FPLC running parameters and protein elution profile. Figure 14B shows the SDS-PAGE (12 %) analysis of 5 μl of each of the fractions A11-B7 collected from the column. The protein gel was stained with coomassie brilliant blue and destained to show the protein bands. Lane M: low-range molecular weight marker (1 μg per band; Bio-Rad), with MW (Daltons): 97,400; 66,200; 45,000; 31,000; 21,500; 14,400.

[0046] Figure 15 shows the purification of the human arginase at 6 h after heat shock (at a higher cell density) by the second 1-ml HiTrap SP FF column. Figure 15A shows the FPLC running parameters and protein elution profile. Figure 15B shows the SDS-PAGE (12 %) analysis of 5 μl of each of the fractions A6-B12 collected from the

column. The protein gel was stained with coomassie brilliant blue and destained to show the protein bands. Lane M: low-range molecular weight marker (1 μg per band; Bio-Rad), with MW (Daltons): 97,400; 66,200; 45,000; 31,000; 21,500; 14,400.

[0047] Figure 16 is the SDS-PAGE (15%) analysis of the human arginase modified with mPEG-SPA (MW 5,000) using the arginase:PEG mole ratio of 1:50. Figure 16A shows the results when reactions were performed on ice. Lane 1: low-range protein marker; Lane 2: arginase (5.35 ug) without PEG added (control); Lane 3: 1 h after reaction; Lane 4: 0.5 h after reaction; Lane 5: 2 h after reaction; Lane 6: 3 h after reaction; Lane 7: 4 h after reaction; Lane 8: 5 h after reaction; Lane 9: 23 h after reaction. Figure 16B shows the results when reactions were performed at room temperature. Lane 1: low-range protein marker; Lane 2: arginase (5.35 ug) without PEG added (control); Lane 3: 1 h after reaction; Lane 4: 0.5 h after reaction; Lane 5: 2 h after reaction; Lane 6: 3 h after reaction; Lane 7: 4 h after reaction; Lane 8: 5 h after reaction; Lane 9: 23 h after reaction.

[0048] Figure 17 is the SDS-PAGE (15%) analysis of the human arginase modified with mPEG-SPA (MW 5,000) using the arginase:PEG mole ratio of 1:20. Figure 17A shows the results when reactions were performed on ice. Lane 1: low-range protein marker; Lane 2: arginase (5.35 μg) without PEG added (control); Lane 3: 1 h after reaction; Lane 4: 0.5 h after reaction; Lane 5: 2 h after reaction; Lane 6: 3 h after reaction; Lane 7: 4 h after reaction; Lane 8: 5 h after reaction; Lane 9: 23 h after reaction. Figure 17B shows the results when reactions were performed at room temperature. Lane 1: low-range protein marker; Lane 2: arginase (5.35 μg) without PEG added (control); Lane 3: 1 h after reaction; Lane 4: 0.5 h after

reaction; Lane 5: 2 h after reaction; Lane 6: 3 h after reaction; Lane 7: 4 h after reaction; Lane 8: 5 h after reaction; Lane 9: 23 h after reaction.

[0049] Figure 18 is the SDS-PAGE (15%) analysis of the human arginase modified with mPEG-CC (MW 5,000). The reactions were performed on ice. Lane 1: low-range protein marker; Lane 2: arginase (5.35 μg) without PEG added (control); Lane 3: 2 h after reaction with arginase:PEG mole ratio of 1:50; Lane 4: empty; Lane 5: 23 h after reaction with arginase:PEG mole ratio of 1:50; Lane 6: 2 h after reaction with arginase:PEG mole ratio of 1:20; Lane 7: 5 h after reaction with arginase:PEG mole ratio of 1:20; Lane 8: 23 h after reaction with arginase:PEG mole ratio of 1:20.

[0050] Figure 19 is the measurement of the isolated recombinant human arginase purity. Figure 19A shows that for Lane 1: 5 μg of purified E. coli-expressed recombinant human arginase obtained from methods described by Ikemoto et al. (Ikemoto et al., 1990, Biochem. J. 270, 697-703). Lane 2: 5 μg of purified B. subtilis-expressed recombinant human arginase obtained from methods described in this report. Figure 19B shows the analysis of densities of protein bands shown in Figure 19A with the Lumianalyst 32 program of Lumi-imager™ (Roche Molecular Biochemicals). Upper panel: results from lane 1 of Figure 19A. Lower panel: results from lane 2 of Figure 19A.

[0051] Figure 20 is a diagram to show the stability of the pegylated modification of the isolated and substantially purified recombinant human arginase *in vitro* in human blood serum.

[0052] Figures 21A and 21B show the half-life determination *in vivo* of mPEG-CC-pegylated isolated and purified recombinant human arginase obtained from

example 8A. Figure 21A shows the *in vivo* activity of the pegylated modification of the recombinant human arginase produced according to the present invention using the activity test described in Example 9A. Figure 21B is a plot from which the first half-life and the second half-life of the pegylated arginase are determined."

[0053] Figures 22A and 22B are schematic diagrams to represent the treatment protocols according to another aspect of the present invention for exogenously administered an pegylated modified isolated and purified recombinant human arginase using daily short infusion (Fig 22A) or continuous infusion (Fig 22B).

[0054] Figure 23 is a schematic diagram to show the treatment protocol of exogenously administered an isolated and purified recombinant human arginase to augment the effect of liver embolization.

DETAILED DESCRIPTION

[0055] In early 2001, three cases of spontaneous transient remission of hepatocellular carcinoma (HCC) were observed by one of the inventors of the present invention. All three patients had spontaneous rupture of HCC with resulting haemoperitoneum. In one case, the plasma arginine was found to be as low as 3 μM and arginine level in the ascitic fluid at 7 μM. These patients all had spontaneous remission of their liver tumour with normalization of AFP after ruptured liver lesions in the absence of any treatment using any pharmaceutical drugs. One patient had remission of his HCC for over 6 months. In accordance with the present invention, it is believed that such remission is caused by the spontaneous release of endogenous arginase into the peritoneum due to the rupture of the liver.

[0056] A series of experiments was designed by the inventor of the present invention to show that endogenous hepatic arginase can be released from the liver after transhepatic arterial embolisation causing systemic arginine deprivation. This has now been filed in the US provisional patent application no. 60/351816, which is incorporated by reference herewith. In the experiment designed by the inventor, small but measurable amount of endogenous hepatic arginase was found to be released into the systemic circulation in patients with unresectable metastatic HCC after hepatic arterial embolisation treatment using lipiodol and gel foam that caused a temporary hepatic perfusion defect. High dose insulin infusion was incorporated into the treatment regime to augment the pharmacological effect of the endogenous arginase released. In a series of 6 cases of HCC treated, 4 had extra hepatic remission of liver cancer suggesting the treatment effects are systemic. One

patient had sustained complete remission, both radiological with CT and PET in his liver and extrahepatic disease (celiac adenopathy). His AFP dropped to normal within 3 weeks and sustained for over 4 months. Interval CT at 4 months showed no demonstrable tumour both hepatic or extrahepatic. The other 3 patients all had remission of their extra hepatic disease (one pulmonary, one mesenteric/retroperitoneal/bone and one retroperitoneal adenopathy) on PET scan at 4 weeks after embolisation. On testing their arginase activities and arginine levels, all had adequate arginine depletion for a period of time lasting from 2 hours to 2 days. In fact the duration of AAD correlated well with the degree and duration of remission of the tumour, both hepatic and extra-hepatic.

[0057] Although the transhepatic arterial embolisation technique was performed in conjunction with high doses of insulin infusion, the inventors, in accordance with the present invention, came to the realisation that the need for the administration of insulin was due to the fact that insufficient arginase activities may be released into the system of the patient such that any protein degradation from the muscle would have a compensatory effect from the arginine deprivation and render the treatment ineffective. In accordance with the present invention, the inventors realised that in order to improve the treatment and to eliminate the need for administration of insulin in conjunction with the arginine deprivation treatment, arginase activity has to be present in sufficiently high amounts in the patient' s system in order to counteract any protein degradation from the muscle. In accordance with the present invention, the inventors therefore set out to produce an arginase enzyme that had sufficiently high enzymatic activities and stability to maintain "adequate arginine deprivation" (hereinafter referred to as "AAD") of below 10 μM in the patient without the need to administrate high dose of insulin.

[0058] Systemic depletion of arginine may cause other undesirable side effect related to nitric oxide deficiency. These include hypertension due to absence of vasodilator effect of NO on vascular endothelium, platelet aggregation and thrombocytopenia secondary to lack of NO and depletion of early clotting factors related to temporary cessation of cell division. The inventor recognized, however, that in nitric oxide knock out mice the animals are not hypertensive and have normal life expectance with normal platelet counts. Thus, in accordance with one aspect of the present invention and in patients with thrombocytopenia, no overt haemorrhagic tendency is seen until platelet count is well below $50,000x10^9$. In patients with thrombotic tendency, therapy entails prolonging the prothrombin time for up to 4x normal.

[0059] The following detailed examples teach how to make and use a highly stable and active recombinant human arginase according to the present invention. Example 1 describes the construction of the recombinant strain of *Bacillus subtilis* LLC101 containing the human arginase I gene. This is followed by two examples of fermentation of the recombinant *B. subtilis*. In the initial fermentation experiments of the recombinant LLC101 cells, batch fermentation and fed-batch fermentation were conducted. It was found that under batch conditions sufficiently high cell density could not be attained. Only under specific fed-batch conditions would cell density be increased to above 10 OD (optical density). These experiments and results are shown in Examples 2A and 2B. A comparison of the 2 fermentation methods is shown in Example 3. Fed-batch fermentation operation was thus chosen for production of isolated and purified recombinant human arginase.

[0060] The LLC101 strain is a heat sensitive strain which causes expression of the recombinant human arginase upon heat shock at 50° C. In the initial optimisation experiments, the heat shock treatment was performed at varying cell densities to obtain the optimal conditions under which maximum isolated and purified recombinant human arginase would be produced. Examples 5 and 6 describe the purification process and the yield of purified arginase thus obtained of two different fed-batch fermentation runs with heat shock at two different OD (optical density at 600 nm), 12.8 and 25. The experimental data showed that although all heat shocks were applied during the exponential growth phase of the LLC101, introduction of heat shock at a lower cell density, e.g., 12.8 OD, produced better results.

[0061] Conditions for maximum expression of isolated and purified recombinant human arginase after heat shock was also optimised by varying the time of harvest after heat shock. Example 4 shows results from harvesting the cells three hours after heat shock and using a fed-batch fermentation process.

[0062] Example 5 describes a purification of isolated and purified recombinant human arginase 6 hours after heat shock at a cell density of 12.8 OD. Example 6 describes the purification of isolated recombinant human arginase 6 hours after heat shock at a higher cell density of 25 OD. Example 7 shows a comparison of the data to compare the yield of the isolated and purified recombinant human arginase under various harvesting and purification conditions. These data show that harvesting cells 6 h after heat shock at a lower cell density of 12.8 produced a higher arginase yield of 132 mg/L.

[0063] The isolated and purified recombinant human arginase was modified to improve stability. Example 8A shows one protocol for the pegylation of the isolated and purified recombinant human arginase using cyanuric chloride (cc) as the cross-linker at an ratio of 1:140 (arginase:PEG). Example 8B describes a different pegylation protocol in which a much lower proportion of cross-linker is added into the reaction mixture with the enzyme. Both cc and succinimide of propionic acid (SPA) were tested as cross-linker. Experimental results show that the method as described in Example 8B using SPA provided a pegylated arginase with a ½-life of 3 days and a specific activity of approximately 255 U/mg as discussed in Example 10.

[0064] Using the method as described above, a highly stable and active recombinant human arginase has been produced. It has sufficiently high activity and stability to allow treatment of patients without significant use of a protein degradation inhibitor because any replenishment of arginine by the muscle would be quickly removed by the systemic isolated and purified recombinant human arginase. Thus, adequate arginine deprivation of below 10 μM can be achieved without high doses of exogenously administered insulin. Various treatment protocols using the isolated and purified recombinant human arginase according to the present invention is described in Examples 11 and 12.

[0065] All references cited above are incorporated by reference herein for the purpose of illustrating the level of skill in the art with respect to the matter discussed. The practice of the invention is exemplified in the following non-limited Examples. The scope of the invention is defined solely by the appended claims, which are in no way limited by the content or scope of the Examples.

EXAMPLES

EXAMPLE 1: CONSTRUCTION OF THE RECOMBINANT STRAIN LLC101

(a) Isolation of the gene encoding human arginase I

[0066] The gene sequence of human arginase I was published in 1987 (Haraguchi, Y. et al., Proc. Natl. Acad. Sci. 84, 412-415, 1987) and primers designed therefrom. Polymerase chain reaction (PCR) was performed to isolate the gene encoding a human arginase using the Expand High Fidelity PCR System Kit (Roche). Primers Arg1 (5' -CCAAACCATATGAGCGCCAAGTCCAGAACCATA-3') (SEQ ID NO: 5) and Arg2 (5' -CCAAACTCTAGAATCACATTTTTTGAATGACATGGACAC-3') (SEQ ID NO: 6), respectively, were purchased from Genset Singapore Biotechnology Pte Ltd. Both primers have the same melting temperature (Tm) of 72 degree C. Primer Arg1 contains a NdeI restriction enzyme recognition site (underlined) and primer Arg2 contains a XbaI site (underlined). These two primers (final concentration 300 nM of each) were added to 5 µl of the human liver 5' -stretch plus cDNA library (Clontech) in a 0.2-ml micro-tube. DNA polymerase (2.6 units, 0.75 µl), the four deoxyribonucleotides (4 µl of each; final concentration 200 µM of each) and reaction buffer (5 µl) and dH_2O (17.75 µl) were also added. PCR was performed using the following conditions: pre-PCR (94 degree C, 5 min), 25 PCR cycles (94 degree C, 1 min; 57 degree C, 1 min; 72 degree C, 1 min), post-PCR (72 degree C, 7 min). PCR product (5 µl) was analyzed on a 0.8% agarose gel and a single band of 1.4 kb was observed. This DNA fragment contains the gene encoding arginase.

(b) Isolation of plasmid pSG1113

[0067] Plasmid pSG1113, which is a derivative of plasmid pSG703 (Thornewell, S. J. et al., 1993, Gene, 133, 47-53), was isolated from the E. coli DH5α clone carrying pSG1113 by using the Wizard Plus Minipreps DNA Purification System (Promega) following the manufacturer' s instruction. This plasmid, which only replicates in E. coli but not in B. subtilis, was used as the vector for the subcloning of the arginase gene.

(c) Subcloning the 1.4 kb PCR product into plasmid pSG1113 to form plasmid pAB101

[0068] The PCR product, prepared using the above protocol, was treated with restriction endonucleases NdeI and XbaI (Promega) in a reaction medium composed of 6 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 150 mM NaCl, 1 mM DTT at 37 degree C for 1.5 h. After completion of the treatment, the reaction mixture was subjected to agarose gel (0.8%) electrophoresis, and the 1.4 kb DNA fragment was recovered from the gel by using the Qiaex II Gel Extraction Kit (Qiagen). Separately, the plasmid pSG1113 was treated with the same restriction endonucleases in the same way. After completion of the treatment, the reaction mixture was subjected to agarose gel (0.8%) electrophoresis, and a DNA fragment having a size of about 3.5 kb was recovered from the gel. This DNA fragment was joined by using T4 DNA ligase to the above 1.4 kb DNA fragment. The ligation mixture was used to transform E. coli XLI-Blue using the conventional calcium method (Sambrook, J. et al., Molecular Cloning, A Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press, New York, 1989) and plated on nutrient agar plate containing 100 μg/ml ampicillin. Colonies were screened for a plasmid with the appropriate insert by restriction analysis. The plasmid constructed

was designated pAB101 (Fig. 1). DNA sequencing was performed with primers Arg1 (SEQ ID NO: 5), Arg2 (SEQ ID NO:6) and Arg6 (5' - CTCTGGCCATGCCAGGGTCCACCC-3') (SEQ_ID NO: 7) to confirm the identity of the gene encoding arginase (Fig. 2).

(d) Construction of the novel recombinant *B. subtilis* prophage strain LLC101

[0069] The plasmid pAB101 was extracted and purified from the clone carrying the pAB101 by using the Wizard Plus Minipreps DNA Purification System (Promega). In the plasmid pAB101, the arginase gene was flanked by the 0.6 kb MunI-NdeI phi-105 phage DNA fragment and the cat gene (Fig. 3). This plasmid DNA (1 μg) was used to transform competent *B. subtilis* 1A304(ϕ105MU331) according to the known method (Anagnostopoulos C. and Spizizen J., 1961. J. Bacteriol. 81, 741-746). The *B. subtilis* strain 1A304(ϕ105MU331) was obtained from J. Errington (Thornewell, S. et al., Gene 133, 47-53, 1993). The strain was produced according to the publications by Thornewell, S. et al., Gene 133, 47-53, 1993 and by Baillie, L. W. J. et al., FEMS Microbiol. Letters 163, 43-47, 1998, which are incorporated herein in their entirety. Plasmid pAB101 (shown linearized) was transformed into the *B. subtilis* strain 1A304 (ϕ105MU331) with selection for the Cm^R marker, and the transformants were screened for an Er^S phenotype. Such transformants should have arisen from a double-crossover event, as shown, placing transcription of the arginase gene (arg) under the control of the strong phage promoters. The thick lines represent the prophage genome, broken lines the *B. subtilis* chromosome, and thin lines plasmid DNA. The genes are shown as shaded arrows pointing in the direction of transcription and translation. Regions of homology are bounded by broken vertical lines and homologous recombination events by 'X' .

[0070] Fifty-two chloramphenicol resistant (Cm^R) colonies were obtained from plating 600 µl of the transformed cells on an agar plate containing chloramphenicol (5 µg/ml). Ten of these colonies were selected randomly and streaked onto an agar plate containing erythromycin (20 µg/ml) and one of these colonies did not grow, indicating that it was erythromycin sensitive (Er^S). This chloramphenicol resistant but erythromycin sensitive colony was thus isolated and named as LLC101. In the chromosome of this newly constructed prophage strain, the erythromycin resistance gene was replaced by the arginase gene by a double crossover event in a process of homologous recombination. The 0.6 kb MunI-NdeI phi-105 phage DNA fragment and the cat gene provided the homologous sequences for the recombination. In this way, the arginase gene was targeted to the expression site in the prophage DNA of *B. subtilis* 1A304(ϕ105MU331) and the arginase gene was put under the control of the strong thermoinducible promoter (Leung, Y. C. and Errington, J., Gene 154, 1-6, 1995).

FERMENTATION OF *B. SUBTILIS* LLC101 CELLS

Example 2A: Batch fermentation in a 2-liter fermentor

[0071] The *B. subtilis* LLC101 strain is maintained in a Nutrient Agar (beef extract 1 g/L, peptone 10 g/L, NaCl 5 g/L and agar 20 g) plate, supplemented with 80 mg/L of ampicillin or 5 mg/L of chloramphenicol when necessary. To prepare the innoculum for batch and fed-batch fermentation, a few colonies of the aforementioned strain were transferred from a freshly prepared Nutrient Agar plate into two 1-L flasks, each containing 80 ml of fermentation medium containing glucose 5 g/L, tryptone 10 g/L, yeast extract 3 g/L, citric acid 1 g/L, KH_2PO_4 1.5 g/L, KH_2PO_4 1.5 g/L, and $(NH_4)_2SO_4$ 3 g/L. The bacterial cell culture was

cultivated at 37 °C and pH 7.0 on an orbital shaker rotating at 250 r.p.m. The cultivation was terminated when OD_{600} reached 5.5-6.0 at about 9-11 h growth time. Then the 160-mL culture broth was introduced into the 2-L fermentor containing 1440-mL fermentation medium (glucose 5 g/L, tryptone 10 g/L, yeast extract 3 g/L, citric acid 1 g/L, KH_2PO_4 1.5 g/L, KH_2PO_4 1.5 g/L, and $(NH_4)_2SO_4$ 3 g/L). The batch fermentation was carried out at a temperature of 37 °C. The pH was controlled at 7.0 by adding sodium hydroxide and hydrochloric acid. The dissolved oxygen concentration was controlled at 20% air saturation with the adjustment of stirring speed. Heat shock was performed at 3.25 h when the culture density (OD_{600nm}) was about 3.9. During the heat shock, the temperature of the fermentor was increased from 37 degree C to 50 degree C and then cooled immediately to 37 degree C. The complete heating and cooling cycle took about 0.5 h. The OD of the culture reached a maximum 6.0 at 3.5 h after heat shock. Cells were harvested for separation and purification of arginase at 9 h after heat shock. The aforementioned strain produced active human arginase in an amount of about 3.0 mg/L of the fermentation medium at 9 h after heat shock. The time-course of the fermentation is plotted in Fig. 4A. The history plot of this batch fermentation showing the changes of parameters such as temperature, stirring speed, pH and dissolved oxygen values is depicted in Fig. 5A.

Example 2B: Fed-batch Fermentation in a 2-liter fermentor

[0072] The Fed-batch fermentation was carried out at 37 degree C, pH 7.0 and dissolved oxygen 20% air saturation. The inoculation procedure was similar to that of the batch fermentation described in Example 2A. Initially, the growth medium was identical to that used in the batch fermentation described in Example

2A. The feeding medium contained 200 g/L glucose, 2.5 g/L $MgSO_4 \cdot 7H_2O$, 50 g/L tryptone, 7.5 g/L K_2HPO_4 and 3.75 g/L KH_2PO_4. The medium feeding rate was controlled with the pH-stat control strategy. In this strategy, the feeding rate was adjusted to compensate the pH increase caused by glucose depletion. This control strategy was first implemented when the glucose concentration decreased to a very low level at about 4.5-h fermentation time. If pH > 7.1, 4 mL of feeding medium was introduced into the fermentor. Immediately after the addition of glucose, the pH value would decrease below 7.1 rapidly. After approximate 10 min, when the glucose added was completely consumed by the bacterial cells, the pH value would increase to a value greater than 7.1, indicating that another 4 mL of feeding medium was due to be added into the fermentor. Heat shock was performed at 5-6 h when the culture density (OD_{600nm}) was between 12.0 and 13.0. During the heat shock, the temperature of the fermentor was increased from 37 degree C to 50 degree C and then cooled immediately to 37 degree C. The complete heating and cooling cycle took about 0.5 h. Cells were harvested for separation and purification of arginase at 3 h and 6 h after heat shock. The aforementioned strain produced active human arginase in an amount of at least about 132 mg per L of the fermentation medium at 6 h after heat shock. The time-course of the fermentation is plotted in Fig. 4B. The history plot of this fed-batch fermentation showing the changes of parameters such as temperature, stirring speed, pH and dissolved oxygen values is indicated in Fig. 5B.

EXAMPLE 3: COMPARISON OF BATCH AND FED-BATCH FERMENTATION

[0073] Table 1 below compares the results of batch and fed-batch fermentation. The comparison demonstrates that the fed-batch fermentation was much superior to the batch operation in terms of culture OD, arginase yield and productivity.

Table 1

	Batch Fermentation	Fed-batch Fermentation
The OD at the start of heat shock	3.9	12.8
Maximum OD reached	6.0	26.8
Arginase Yield (mg/L)	3.0	132
Arginase Productivity (mg/L-h)	0.25	11.2

EXAMPLE 4: PURIFICATION OF ARGINASE AT 3 H AFTER HEAT SHOCK AFTER FED-BATCH FERMENTATION AT LOW CELL DENSITY

[0074] Fed-batch fermentation in a 2-liter fermentor was performed as described in Example 2B. The cell density of the fed-batch culture was monitored at 30 or 60 min interval and the temperature of the culture raised to 50° C for heat shock at 5.5 hours after the fermentation started when the OD of the culture reached 12.8 (see Fig. 4B and Fig. 5B).

[0075] The cell culture (470 ml) collected at 3 h after heat shock was centrifuged at 5,000 rpm for 20 min at 4 degree C to pellet the cells. The wet weight of the cells was 15.1 g. The culture supernatant liquor was discarded and the cell pellet was stored at –80 degree C. The cells are stable at this temperature for a few days. To extract intracellular proteins, the cell pellet was resuspended in 140 ml solubilization buffer 50 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 5 mM $MnSO_4$, lysozyme (75 μg/ml). After incubation at 30 degree C for 15 min, the mixture was sonicated for eight times, each time lasted for 10 s (the total time was 80 s), at 2 min intervals using the Soniprep 150 Apparatus (MSE). About 500 units of

deoxyribonuclease I (Sigma D 4527) was added and the mixture was incubated at 37 degree C for 10 min to digest the chromosomal DNA. After centrifugation at 10,000 rpm for 20 min at 4 degree C, the supernatant, containing the crude protein extract, was assayed for the presence of the arginase activity and analyzed by SDS-PAGE (Laemmli, 1970, Nature, 227, 680-685).

[0076] A 5-ml HiTrap Chelating column (Pharmacia) was equilibrated with 0.1 M $NiCl_2$ in dH_2O, for 5 column volumes. The crude protein extract (140 ml) was loaded onto the column. Elution was performed with a linear gradient (0-100%) at a flow rate of 5 ml/min for 15 column volumes under the following conditions: Buffer A = start buffer 0.02 M sodium phosphate buffer (pH 7.4), 0.5 M NaCl; Buffer B = start buffer containing 0.5 M imidazole. The elution profile is shown in Fig. 6A and the protein gel is shown in Fig. 6B. Fractions 13-20 were pooled (16 ml) and diluted ten times with start buffer 0.02 M sodium phosphate buffer (pH 7.4), 0.5 M NaCl. This was loaded onto a second 5-ml HiTrap Chelating column (Pharmacia), repeating the same procedure as above. The elution profile is shown in Fig. 7A and the protein gel is shown in Fig. 7B. Fractions 12-30 containing arginase were pooled (38 ml) and salt was removed using a 50-ml HiPrep 26/10 desalting column (Pharmacia) with the following conditions: flow rate = 10 ml/min, buffer = 10 mM Tris-HCl (pH 7.4) and length of elution = 1.5 column volume. The protein concentration was measured by the method of Bradford (Bradford, M. M., , Anal. Biochem., 72, 248-254, 1976). A total of 56.32 mg of arginase was purified from 470 ml cell culture. The yield of purified arginase was estimated to be 119.8 mg/l cell culture or 3.73 mg/g wet cell weight.

EXAMPLE 5: PURIFICATION OF ARGINASE AT 6 H AFTER HEAT SHOCK AFTER FED-BATCH FERMENTATION AT LOW CELL DENSITY

[0077] Fed-batch fermentation in a 2-liter fermentor was performed as described in Example 4. The cell culture (650 ml) collected at 6 h after heat shock at OD 12.8 was centrifuged at 5,000 rpm for 20 min at 4 degree C to pellet the cells. The wet weight of the cells was 24 g. The culture supernatant liquor was discarded and the cell pellet was stored at –80° C. The cells are stable at this temperature for a few days. To extract intracellular proteins, the cell pellet was resuspended in 140 ml solubilization buffer 50 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 5 mM $MnSO_4$, lysozyme (75 μg/ml). After incubation at 30 degree C for 15 min, the mixture was sonicated for eight times, each time lasted for 10 s (the total time was 80 s), at 2 min intervals using the Soniprep 150 Apparatus (MSE). About 500 units of deoxyribonuclease I (Sigma D 4527) was added and the mixture was incubated at 37 degree C for 10 min to digest the chromosomal DNA. After centrifugation at 10,000 rpm for 20 min at 4 degree C, the supernatant, containing the crude protein extract, was assayed for the presence of the arginase activity and analyzed by SDS-PAGE (Laemmli, 1970, Nature, 227, 680-685).

[0078] A 5-ml HiTrap Chelating column (Pharmacia) was equilibrated with 0.1 M $NiCl_2$ in dH_2O, for 5 column volumes. The crude protein extract (140 ml) was loaded onto the column. Elution was performed with a linear gradient (0-100%) at a flow rate of 5 ml/min for 15 column volumes under the following conditions: Buffer A = start buffer 0.02 M sodium phosphate buffer (pH 7.4), 0.5 M NaCl; Buffer B = start buffer containing 0.5 M imidazole. The elution profile is shown in Fig. 8A and the protein gel is shown in Fig. 8B. Fractions 13-24 were pooled (24 ml) and diluted ten times with start buffer 0.02 M sodium phosphate buffer (pH 7.4), 0.5 M NaCl. This was

loaded onto a second 5-ml HiTrap Chelating column (Pharmacia), repeating the same procedure as above. The elution profile is shown in Fig. 9A and the protein gel is shown in Fig. 9B. Fractions 12-24 containing arginase were pooled (26 ml) and salt was removed using a 50-ml HiPrep 26/10 desalting column (Pharmacia) with the following conditions: flow rate = 10 ml/min, buffer = 10 mM Tris-HCl (pH 7.4) and length of elution = 1.5 column volume. The protein concentration was measured by the method of Bradford (Bradford, M. M., 1976, Anal. Biochem., 72, 248-254). A total of 85.73 mg of arginase was purified from 650 ml cell culture. The yield of purified arginase was estimated to be 132 mg/l cell culture or 3.57 mg/g wet cell weight.

EXAMPLE 6: PURIFICATION OF ARGINASE AT 6 H AFTER HEAT SHOCK AT A HIGHER CELL DENSITY

[0079] In this particular fed-batch fermentation, the process was similar to the above example except that the heat shock was performed at 8 h when the culture density (OD_{600nm}) was about 25. During the heat shock, the temperature of the fermentor was increased from 37 degree C to 50 degree C and then cooled immediately to 37 degree C. The complete heating and cooling cycle took about 0.5 h. A portion of the cell culture (760 ml) was harvested for separation and purification of arginase at 6 h after heat shock. The time-course of bacterial cell growth in this fermentation is plotted in Fig. 10. The history plot of this fed-batch fermentation showing the changes of parameters such as temperature, stirring speed, pH and dissolved oxygen values is indicated in Fig. 11.

[0080] The cell culture (760 ml) collected at 6 h after heat shock was centrifuged at 5,000 rpm for 20 min at 4 degree C to pellet the cells. The wet weight of the cells was 32 g. The culture supernatant liquor was discarded and the cell pellet was stored at –80 degree C. The cells are stable at this temperature for a few days. To

extract intracellular proteins, the cell pellet was resuspended in 280 ml solubilization buffer 50 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 5 mM $MnSO_4$, lysozyme (75 μg/ml). After incubation at 30 degree C for 15 min, the mixture was sonicated for eight times, each time lasted for 10 s (the total time was 80 s), at 2 min intervals using the Soniprep 150 Apparatus (MSE). About 500 units of deoxyribonuclease I (Sigma D 4527) was added and the mixture was incubated at 37 degree C for 10 min to digest the chromosomal DNA. After centrifugation at 10,000 rpm for 20 min at 4 degree C, the supernatant, containing the crude protein extract, was assayed for the presence of the arginase activity and analyzed by SDS-PAGE (Laemmli, 1970, Nature, 227, 680-685).

[0081] A 5-ml HiTrap Chelating column (Pharmacia) was equilibrated with 0.1 M $NiCl_2$ in dH_2O, for 5 column volumes. The crude protein extract (280 ml) was loaded onto the column. Elution was performed with a linear gradient (0-100%) at a flow rate of 5 ml/min for 15 column volumes under the following conditions: Buffer A = start buffer 0.02 M sodium phosphate buffer (pH 7.4), 0.5 M NaCl; Buffer B = start buffer containing 0.5 M imidazole. The elution profile is shown in Fig. 12A and the protein gel is shown in Fig. 12B. Fractions 17-31 were pooled (30 ml) and diluted ten times with start buffer 0.02 M sodium phosphate buffer (pH 7.4), 0.5 M NaCl. This was loaded onto a second 5-ml HiTrap Chelating column (Pharmacia), repeating the same procedure as above. The elution profile is shown in Fig. 13A and the protein gel is shown in Fig. 13B. Fractions 10-20 containing arginase were pooled (22 ml) and salt was removed using a 50-ml HiPrep 26/10 desalting column (Pharmacia) with the following conditions: flow rate = 10 ml/min, buffer = 10 mM Tris-HCl (pH 7.4) and length of elution = 1.5 column volume. The sample was then loaded onto a 1-ml HiTrap SP FF column (Pharmacia). Elution

was performed with the following conditions: flow rate = 1 ml/min, Buffer A = 10 mM Tris-HCl (pH 7.4), Buffer B = 10 mM Tris-HCl (pH 7.4) containing 1 M NaCl, linear gradient (0-100%), length of elution = 30 column volumes. The elution profile is shown in Fig. 14A and the protein gel is shown in Fig. 14B. Fractions A12-B7 were pooled (7 ml) and diluted ten times with start buffer 0.02 M sodium phosphate buffer (pH 7.4), 0.5 M NaCl. This sample was loaded onto a second 1-ml HiTrap SP FF column (Pharmacia), repeating the same procedure as above, except that the elution was performed with a segmented gradient. The elution profile is shown in Fig. 15A and the protein gel is shown in Fig. 15B. Fractions A7-B12 were pooled (7 ml) and desalted as above using a 50-ml HiPrep 26/10 desalting column (Pharmacia). The protein concentration was measured by the method of Bradford (Bradford, M. M., 1976, Anal. Biochem., 72, 248-254). A total of 41.61 mg of arginase was purified from 760 ml cell culture. The yield of purified arginase was estimated to be 55.5 mg/l cell culture or 1.3 mg/g wet cell weight.

EXAMPLE 7: COMPARISON OF YIELD OF ISOLATED AND PURIFIED RECOMBINANT HUMAN ARGINASE HARVESTED AND PURIFIED UNDER VARIOUS CONDITIONS

[0082] Table 2 below compares the yield of the isolated and purified recombinant human arginase produced under various harvesting and purification conditions. These data show that harvesting cells 6 h after heat shock at a lower cell density of 12.8 produced a higher arginase yield of 132 mg/L.

Table 2

Fed-batch Fermentation	Arginase Yield (mg/L)	
	Harvested 3 h after heat shock	Harvested 6 h after heat shock
Heat shock at OD 12.8	120	132
Heat shock at OD 25	-	55.5

EXAMPLE 8A: PREPARATION OF THE PEGYLATED ENZYME USING CYANURIC CHLORIDE (CC)

[0083] 50 mg Arginase was dissolved in 20 ml PBS buffer solution (pH 7.4) to a final concentration of 2.5 mg/ml. Heat activation of arginase was carried out at 60°C for 10 minutes. After activation, the temperature of the enzyme was allowed to bring back to room temperature. 1 g cyanuric chloride activated polyethylene glycol (mPEG-CC) (MW=5000, Sigma) was added to arginase at mole ratio 1: 140 (arginase:PEG). A magnetic stirring bar was used to stir the mixture until all of the polyethylene glycol (PEG) was dissolved.

[0084] When all of the PEG was dissolved, pH of the PEG-arginase mixture was adjusted to 9.0 with 0.1 N NaOH, pH was further maintained at 9.0 for the next 30 minutes with further additions of NaOH. Pegylation was stopped by adjusting pH back to 7.2 with addition of 0.1 N HCl.

[0085] The pegylated arginase was dialyzed against 2-3 liters of PBS buffer solution, pH 7.4, at 4°C, with the use of a Hemoflow F40S capillary dialyzer (Fresenius Medical Care, Germany) to remove excess PEG. After dialysis, pegylated arginase was recovered and the final concentration was readjusted.

[0086] The pegylated arginase was filtered through a 0.2 μ m filter into a sterilized container and was stored at 4°C. The ½-life of this enzyme in a human patient was tested to be about 6 hours (see Fig. 21).

EXAMPLE 8B: PREPARATION OF PEGYLATED ISOLATED AND PURIFIED RECOMBINANT HUMAN ARGINASE EXPRESSED IN B. SUBTILIS AND USING EITHER CC OR SPA AT A LOWER PEG RATIO

[0087] Pegylation was first developed by Davis, Abuchowski and colleagues (Davis, F. F. et al., 1978, Enzyme Eng. 4, 169-173) in the 1970s. In contrast to modifying the formulation of a drug, chemical attachment of poly(ethylene glycol) PEG moieties to therapeutic proteins (a process known as "pegylation") represents a new approach that may enhance important drug properties (Harris, J. M. et al., Clin. Pharmacokinet. 40, 539-551, 2001).

[0088] In 1979, Savoca et al. attached methoxypolyethylene glycol (mPEG) of 5,000 Daltons covalently to bovine liver arginase using 2,4,6-trichloro-s-triazine (cyanuric chloride) as the coupling agent (Savoca, K. V. et al., Biochimica et Biophysica Acta 578, 47-53, 1979). The conjugate (PEG-arginase) only retained 65% of its original enzymatic activity. They reported that the blood-circulating life of PEG-arginase in mice was extended over that of bovine arginase. The half-life of injected bovine arginase was less than 1 h, whereas that of the PEG-enzyme was 12 h. Their data also indicated that bovine arginase modified by PEG was rendered both non-immunogenic and non-antigenic when tested in mice.

[0089] Recombinant human arginase (1.068 mg) was dissolved in 125 mM borate buffer solution (pH 8.3) on ice or at room temperature. Activated PEG, succinimide of mPEG propionic acid (mPEG-SPA; MW 5,000) or mPEG activated with cyanuric chloride (mPEG-CC; MW 5,000), was added into the solution at arginase:PEG mole ratios of 1:50 or 1:20. This was performed in two stages. At the first stage, half of the PEG was added into the arginase solution little by little and

mixed for 30 min gently on ice to prevent the pH getting out of the recommended range of 8.0-8.5. The other half of the PEG was added to this solution and further mixed gently for 0.5-23 h. The mixture was then dialyzed against dH_2O by changing with dH_2O at least 3 times at 4 degree C using dialysis membrane with cut-off value of below 10,000. Both mPEG-SPA and mPEG-CC use amino groups of lysines and the N-terminus of the protein as the site of modification.

[0090] When human arginase was modified on ice or at room temperature with mPEG-SPA (MW 5,000) using an arginase:PEG mole ratio of 1:50, most of the enzyme molecules were modified after 1 h of reaction (Fig. 16). The sample appeared the same even after 23 h of reaction. Human arginase molecules were attached with different numbers of PEG molecules and generated molecules of various molecular weights. As expected, when a lower mole ratio of 1:20 was used for the pegylation reaction, a higher proportion of arginase was found in the non-pegylated form (Fig. 17). However, for both of the mole ratios of arginase:PEG used, longer reaction time and the use of room temperature instead of ice did not seem to affect the extent of pegylation. With mPEG-SPA (MW 5,000), a mole ratio of 1:50 and 1 h of reaction, the pegylated human arginase retained as much as 72-76% of its original enzymatic activity (see Table 3 below), which is higher than that reported for the bovine arginase (Savoca, K. V. et al., 1979, Biochimica et Biophysica Acta 578, 47-53).

[0091] When human arginase was modified on ice with mPEG-CC (MW 5,000) using an arginase:PEG mole ratio of 1:50, the reaction was quite slow and it took 23 h to complete the pegylation (Fig. 18). Moreover, most of the enzyme molecules were converted to a narrow spectrum of very high molecular weights.

The reaction was much slower if a lower mole ratio of 1:20 was used, as indicated in Fig. 18.

Table 3. Activity (%) of arginase when pegylated with various activated PEG at different mole ratios and temperatures.

Time (h) allowed for the pegylation reaction	Activity (%) of arginase (pegylated at room temperature with arginase: mPEG-SPA ratio of 1:20)	Activity (%) of arginase (pegylated on ice with arginase:mPEG-SPA ratio of 1:20)	Activity (%) of arginase (pegylated at room temperature with arginase: PEG-SPA ratio of 1:50)	Activity (%) of arginase (pegylated on ice with arginase:mPEG-SPA ratio of 1:50)	Activity (%) of arginase (pegylated on ice with arginase:mPEG-CC ratio of 1:20)	Activity (%) of arginase (pegylated on ice with arginase:mPEG-CC ratio of 1:50)
0	100	100	100	100	100	100
1	83	76	76	72	ND	ND
2	79	76	72	68	68	64
5	83	74	74	72	65	65
23	75	72	72	64	66	66

ND: Not determined.

100% activity of arginase is equivalent to 336 units/mg of protein.

EXAMPLE 9A: ½-LIFE DETERMINATION *in vivo* OF mPEG-CC-PEGYLATED ISOLATED AND PURIFIED RECOMBINANT HUMAN ARGINASE OBTAINED FROM EXAMPLE 8A

[0092] The pegylated isolated and purified recombinant human arginase was injected into a patient. A 3 ml blood sample in EDTA was taken from patient on a daily basis. The tube was pre-cooled to 4°C on melting ice to prevent ex-vivo enzymatic reaction. The blood was then immediately spun down at 14000 rpm for 2 minutes to remove red blood cells. 1.5 ml supernatant (plasma) was pipetted out and transferred to a new eppendorf tube. The plasma was then incubated at 37°C for 30 minutes. After incubation, arginine was added as a substrate in concentration of 100 µM. Enzyme reaction was carried out at 37°C for 0, 10, 30, 60 minutes. At each time interval, reaction was stopped by taking out 300 µl reaction sample to a new eppendorf tube containing 300 µl 10% trichloroacetic acid. Samples were taken and spun at maximum speed (14000 rpm) for 10 minutes. Supernatant was pipetted out and filtered with 0.45 µm filter. Finally, samples at different time intervals were analysed with amino acid analyzer (Hitachi, L8800). The results are shown in Fig. 21A.

[0093] Two batches of pegylated arginase were prepared as described in Example 8A during the studies. The first batch of pegylated arginase was prepared with arginase:PEG mole ratio of 1:140. The second batch of pegylated arginase was prepared with arginase:PEG mole ratio of 1:70. The pegylation protocol and condition used for preparing the two batches were identical (see Example 8A).

[0094] At time zero, 50 mg of the first batch of pegylated arginase was infused. After 12 hours, another 50 mg of the first batch of pegylated arginase was infused. The third arginase infusion was done at hour 24 during which another 50 mg of the first batch of pegylated arginase was used.

[0095] From hour 26 to hour 72, continuous infusion of the first batch of pegylated arginase (100 mg/day) was carried out instead of intermittent infusion (50 mg/dosage). From hour 72 to hour 144, continuous infusion of the second batch of pegylated arginase was carried out at a rate of 100 mg/day. Continuous arginase infusion was stopped at hour 144, and the measurement of the half-life started from this point. The results of the half-life determination are shown in Fig. 21B. Time zero in Fig. 21B is equivalent to hour 144 in Fig. 21A.

[0096] The results suggested that the half-life of the activity of the recombinant human arginase could be divided into two phases. The first half-life of the pegylated enzyme was about 6 hours. It took about 6 hours to reduce the relative activity from 100% to 50% (see Fig. 21B). However, the second half-life was about 21 days. It took about 21 days to reduce the relative activity from 50% to 25%. This dual half-life effects might be due to a number of factors including the use of higher amount of mPEG-CC in the pegylation and the specific infusion protocol used.

EXAMPLE 9B: ½ LIFE DETERMINATION OF mPEG-SPA-PEGYLATED ISOLATED AND PURIFIED RECOMBINANT HUMAN ARGINASE IN USING THE METHOD IN HUMAN BLOOD SERUM

[0100] Purified recombinant human arginase (1 mg) was dissolved in 1 ml of 125

mM borate buffer solution (pH 8.3) on ice. Activated PEG (mPEG-SPA, MW 5,000) (7.14 mg) was added into the protein solution slowly at a mole ratio of arginase: PEG = 1 : 50. The mixture was stirred on ice for 2.5 h, following the method as described in Example 8B.

[0101] Pegylated arginase (305.6 □l) at a concentration of 1 mg/ml was added into human blood serum (1 ml) and the final concentration of pegylated arginase was 0.24 mg/ml. The mixture was divided into 20 aliquots in eppendorf tubes (65 □l mixture in each eppendorf tube) and then incubated at 37°C. A 1-2 □l portion of the mixture from each eppendorf tube was used to test the arginase activity. Results are shown in Figure 20. The ½-life was determined to be approximately 2 – 3 days.

EXAMPLE 10: CHARACTERIZATION OF B. SUBTILIS-EXPRESSED ISOLATED AND PURIFIED RECOMBINANT HUMAN ARGINASE AND PEGYLATED ISOLATED AND PURIFIED RECOMBINANT HUMAN ARGINASE

[0102] Purified *E. coli*-expressed recombinant human arginase obtained from methods described by Ikemoto et al. (Ikemoto et al., Biochem. J. 270, 697-703, 1990) was compared to purified *B. subtilis*-expressed recombinant human arginase obtained from methods described of the present invention (Fig. 19A and B). Analysis of densities of protein bands shown in Fig. 19A with the Lumianalyst 32 program of Lumi-imager™ (Roche Molecular Biochemicals) indicated that the process developed in the present invention produced up to 100% pure recombinant human arginase (Fig. 19B). In the preferred embodiment, recombinant arginase of 80-100% purity is used. In the most preferred embodiment, the recombinant

arginase according to the present invention is 90-100% pure using SDS-PAGE followed by lumi-imaging.

[0103] The rate of the release of urea from L-arginine by arginase was monitored in a system containing urease, L-glutamate dehydrogenase and NADPH (Ozer, N. Biochem. Med. 33, 367-371, 1985). To prepare the master mix, 0.605 g Tris, 0.0731 g α-ketoglutarate and 0.4355 g arginine were dissolved in 40 ml dH_2O. The pH was adjusted to 8.5 with 1 M HCl and then 0.067 g urease was added to the mixture. The pH was further adjusted to 8.3 with HCl before 0.0335 g glutamate dehydrogenase and 0.0125 g NADPH was added. The final volume was adjusted to 50 ml with dH_2O to form the master mix. The master mix (1 ml) was pipetted into a quartz cuvette. For measuring arginase activity, 1-5 μg arginase was added and the decrease in absorbance at 340 nm (A_{340}) was followed for 1-3 min at 30 degree C. One unit of arginase was defined as the amount of enzyme that released 1 μmol of urea for 1 min under the given conditions. The specific activity of the purified recombinant human arginase of the present invention was calculated to be 336 units/mg of protein, which was in the same order of magnitude as the reported values for purified human erythrocyte arginase (204 units/mg of protein; Ikemoto et al., Ann. Clin. Biochem. 26, 547-553, 1989) and the *E. coli*-expressed isolated and purified recombinant human arginase (389 units/mg of protein; Ikemoto et al., Biochem. J. 270, 697-703, 1990).

[0104] With mPEG-SPA (MW 5,000), a mole ratio of 1:50 and 1 h of reaction, the pegylated human arginase retained as much as 72-76% of its original enzymatic activity (see Table 3). That means the specific activity of the pegylated human arginase was 242-255 U/mg.

[0105] With mPEG-CC (MW 5,000), the pegylated human arginase retained 64-68% of its original enzymatic activity (Table 3), similar to that of the pegylated bovine arginase (Savoca, K. V. et al., , Biochimica et Biophysica Acta 578, 47-53, 1979).

EXAMPLE 11: TREATMENT PROTOCOL USING EXOGENOUSLY ADMINISTERED ISOLATED AND PURIFIED RECOMBINANT HUMAN ARGINASE

[0106] Blood samples of patients are taken twice daily throughout treatment for arginine levels, arginase activities, complete blood picture and full clotting profile. Renal and liver function are taken at least every other days, sooner if deemed necessary.

[0107] Vital signs (BP, Pulse, Respiratory rate, Oximeter reading) are taken every 15 minute for 1 hour after commencement of arginase infusion then hourly until stable. Thereafter, at the discretion of the treating physician.

[0108] 20 minutes before arginase infusion, premedication with dipheneramine 10mg iv. and hydrocortisone 100mg iv. to be given before each fresh infusion of arginase.

[0109] On day One (induction period), isolated and purified recombinant human arginase is infused over 1 hour at 10 a.m. and repeated at night at 10 p.m. as shown in Figures 22A and 22B.

[0110] From day 2 - 5, arginase at 2 mg/kg is given in short daily infusions as shown in Figure 22A.

[0111] In a second method, the isolated and purified recombinant human arginase may be administered as continuous infusion over several days after initial induction, as shown in Figure 22B.

EXAMPLE 12: TREATMENT PROTOCOL USING EXOGENOUSLY ADMINISTERED ARGINASE TO AUGMENT LIVER EMBOLIZATION TREATMENT

[0112] In this example, a liver cancer patient is treated with the standard liver embolization procedure using, for example, Lipiodol and gel foam. This reduces the oxygen and nutrient supply to the liver tumour by blocking the hepatic artery blood flow into the liver. Isolated and purified recombinant human arginase is then administered to augment the endogenous arginase released by the traumatized liver. No exogenously administered insulin is required for non-diabetic patients with this treatment using the isolated and purified recombinant human arginase with a ½-life of approximately 2-3 days and high activity of approximately 255 U/mg as described in Example 9B. The schematic treatment protocol is shown in Figure 23.

[0113] It must be noted that as used herein and in the appended claims, the singular forms "a," "and," and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, reference to "a pharmaceutical preparation" includes mixtures of different preparations and reference to "the method of treatment" includes reference to equivalent steps and methods known to those skilled in the art, and so forth.

[0114] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar to

equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to describe and disclose specific information for which the reference was cited in connection with.

[0115] The invention having been fully described, modifications within its scope will be apparent to those of ordinary skill in the art. All such modifications are within the scope of the invention.

CLAIMS

1. An isolated recombinant human arginase having a purity of 80-100%.

2. An isolated and substantially pure recombinant human arginase according to claim 1 wherein said enzyme has substantially the same nucleotide sequence as set forth in SEQ ID NO: 1 or SEQ ID NO: 2, wherein said nucleotide sequence encodes an amino acid sequence as set forth in SEQ ID NO: 3 of said human arginase, wherein said isolated recombinant human arginase has a substantially high enzyme activity of at least 250 U/mg.

3. An isolated and substantially pure recombinant human arginase according to claim 1 wherein said enzyme is modified to have sufficient stability in serum or plasma with a half-life of approximately at least 3 days.

4. The isolated and substantially purified recombinant human arginase according to claim 1 is human arginase I.

5. The isolated and substantially purified recombinant human arginase according to claim 1 is cloned and expressed in a bacterial strain selected from the group consisting of *E Coli*, *bacilli*, *enterobacteriaceae*, *Pseudomonas* and the like.

6. The isolated and substantially purified recombinant human arginase according to claim 5, wherein said bacterial strain is *Bacillus subtilis*.

7. The isolated and substantially purified recombinant human arginase according to claim 1, wherein said modification is pegylation.

8. The isolated and substantially purified recombinant human arginase according to claim 7, wherein said pegylation results from covalently

attaching at least one polyethylene glycol (PEG) moiety to said enzyme using a coupling agent.

9. The isolated and substantially purified recombinant human arginase according to claim 7; wherein said coupling agent is selected from the group consisting of 2,4,6-trichloto-s-triazine (cyanuric chloride, CC) and succinimide propionic acid (SPA).

10. A method of isolation and purification of said recombinant human arginase, comprising:

(a) cloning a gene encoding human arginase I;

(b) constructing a recombinant *B. substilis* prophase strain for expression of said cloned human arginase I;

(c) fermenting *B. substilis* cells with said constructed recombinant *B. substilis* prophase strains; and

(d) purifying said cloned human arginase I after heat shock.

11. The method according to claim 10, further comprising modifying said purified recombinant human arginase I with pegylation that covalently attaches at least one polyethylene glycol (PEG) moiety to said recombinant human arginase using a coupling agent.

12. The method according to claim 11, wherein said polyethylene glycol moieties are methoxypolyethylene glycol (mPEG).

13. The method according to claim 11, wherein said coupling agent is selected from the group consisting of 2,4,6-trichloto-s-triazine (cyanuric chloride, CC) and succinimide propionic acid (SPA).

14. A pharmaceutical composition comprising an isolated and substantially purified recombinant human arginase.

15. The pharmaceutical composition according to claim 14, wherein said recombinant human arginase is human arginase I.

16. The pharmaceutical composition according to claim 14, wherein said composition is further formulated in a form selected from the group consisting of a solid, a solution, an emulsion, a dispersion, a micelle, a liposom, and the like.

17. The pharmaceutical composition according to claim 16, wherein said formulation of said pharmaceutical composition is in a form suitable for oral use, or for a sterile injectable solution or suspension.

18. The pharmaceutical composition according to claim 17, wherein said pharmaceutical composition is administered to a patient, wherein said recombinant human arginase in said pharmaceutical composition is an active ingredient having a specific enzyme activity of at least 250 U/mg, and wherein said recombinant human arginase in said pharmaceutical composition maintains a systemic level of arginine in said patient of less than about 10 μM for at least 3 days.

19. The pharmaceutical composition according to claim 18, wherein said recombinant human arginase has a half-life in said patient plasma of approximately at least 3 days.

20. The pharmaceutical composition according to claim 18, wherein said recombinant human arginase has a half-life in said patient plasma of approximately at least 1 days.

21. The pharmaceutical composition according to claim 18, wherein said half-life in said patient plasma is measured *in vivo* or *in vitro*.

22. The pharmaceutical composition according to claim 18, wherein said pharmaceutical composition is administered in said patient in short daily infusions of not more than 1 hour.

23. The pharmaceutical composition according to claim 18, wherein said patient has liver diseases.

24. The pharmaceutical composition according to claim 18, wherein said patient has cancer.

25. The pharmaceutical composition according to claim 18, wherein said patient is administered said pharmaceutical composition only as a single pharmaceutical agent, wherein no other protein breakdown inhibitor needs to be administered in said patient.

26. The pharmaceutical composition according to claim 25, wherein said protein breakdown inhibitor is insulin.

27. The use of human arginase for the preparation of a medicament.

28. The use according to claim 27 wherein said medicament is used for the treatment of human malignancies.

29. The use according to claim 27 wherein said human arginase has a purity of 80-100%.

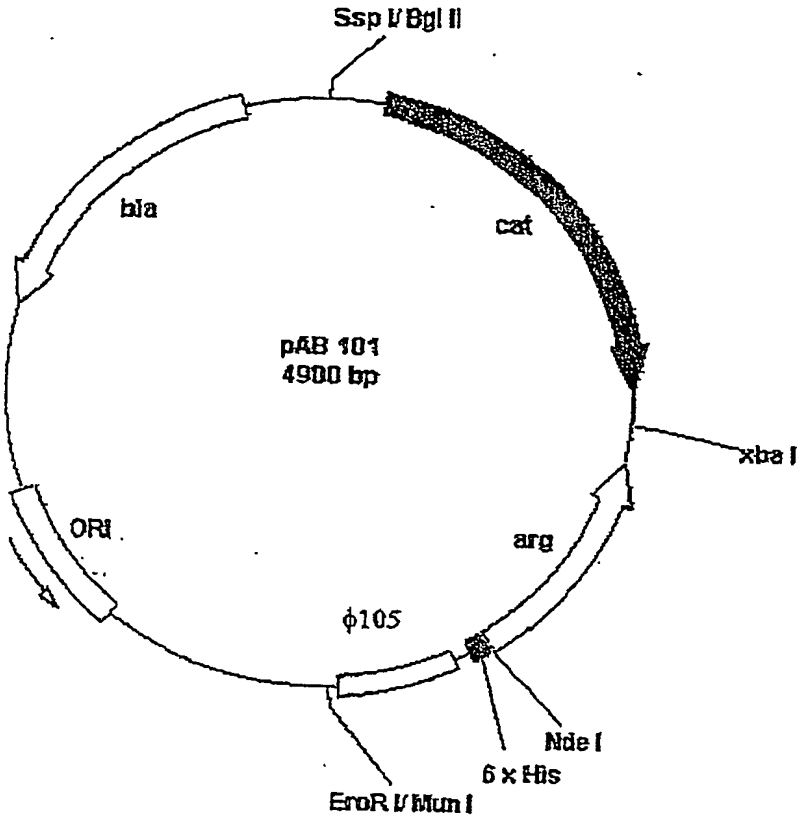
30. The use according to claim 27 wherein said human arginase has an enzymatic activity of at least 250 U/mg.

31. The use of human arginase according to claim 27 wherein said human arginase has sufficient stability in human to have a half-life of approximately 3 days.

ABSTRACT

The present invention provides an isolated and substantially purified recombinant human arginase having sufficiently high enzymatic activity and stability to maintain AAD in a patient. The present invention also provides a pharmaceutical composition comprising the modified invention enzyme and method for treatment of diseases using the invention pharmaceutical composition.

Fig. 1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bla
cat
pAB 101
4900 bp
xba I
ORI
arg
ϕ105
Nde I
6 x His
EroR I/Mun I

Fig. 2A

```
   1 gaattgtacg tcaaagagat gaagcagaaa aacgtcgtcg agaagaagct gaacgacaaa
  61 aagtgaaatg cgagggaagt ccaagaaatg gtgattatga gggtgtctat ttcaccaaaa
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 541 ggatagacaa tcggcggtta acggcttgag tgcgggggca gtttagaaag aatatgattg
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 661 gattattgga gctccttttct caaagggaca gccacgagga ggggtggaag aaggccctac
 721 agtattgaga aaggctggtc tgcttgagaa acttaaagaa caagagtgtg atgtgaagga
 781 ttatggggac ctgccctttg ctgacatccc taatgacagt ccctttcaaa ttgtgaagaa
 841 tccaaggtct gtgggaaaag caagcgagca gctggctggc aaggtggcac aagtcaagaa
 901 gaacggaaga atcagcctgg tgctgggcgg agaccacagt ttggcaattg gaagcatctc
 961 tggccatgcc agggtccacc ctgatcttgg agtcatctgg gtggatgctc acactgatat
1021 caacactcca ctgacaacca caagtggaaa cttgcatgga caacctgtat ctttcctcct
1081 gaaggaacta aaaggaaaga ttcccgatgt gccaggattc tcctgggtga ctccctgtat
1141 atctgccaag gatattgtgt atattggctt gagagacgtg gaccctgggg aacactacat
1201 tttgaaaact ctaggcatta aatacttttc aatgactgaa gtggacagac taggaattgg
1261 caaggtgatg gaagaaacac tcagctatct actaggaaga aagaaaaggc caattcatct
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1801 tatattttct aacttggcaa aagacttatc cttagaaaga gaagtgtaca ttgatttcca
1861 attaaaaatt tgctggcatt aaaaataagc acacttacat aagcccccat acatagagtg
1921 ggactcttgg aatcaggaga caaagctacc acatgtggaa aggtactatg tgtccatgtc
1981 attcaaaaaa tgtgattcta ga
```

Fig. 2B

```
                          1  atgcatcaccatcaccatcat
                             M  H  H  H  H  H  H
   22 atgagcgccaagtccagaaccatagggattattggagctcctttc
      M  S  A  K  S  R  T  I  G  I  I  G  A  P  F
   67 tcaaagggacagccacgaggaggggtggaagaaggccctacagta
      S  K  G  Q  P  R  G  G  V  E  E  G  P  T  V
  112 ttgagaaaggctggtctgcttgagaaacttaaagaacaagagtgt
      L  R  K  A  G  L  L  E  K  L  K  E  Q  E  C
  157 gatgtgaaggattatggggacctgccctttgctgacatccctaat
      D  V  K  D  Y  G  D  L  P  F  A  D  I  P  N
  202 gacagtccctttcaaattgtgaagaatccaaggtctgtgggaaaa
      D  S  P  F  Q  I  V  K  N  P  R  S  V  G  K
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      A  S  E  Q  L  A  G  K  V  A  Q  V  K  K  N
  292 ggaagaatcagcctggtgctgggcggagaccacagtttggcaatt
      G  R  I  S  L  V  L  G  G  D  H  S  L  A  I
  337 ggaagcatctctggccatgccagggtccaccctgatcttggagtc
      G  S  I  S  G  H  A  R  V  H  P  D  L  G  V
  382 atctgggtggatgctcacactgatatcaacactccactgacaacc
      I  W  V  D  A  H  T  D  I  N  T  P  L  T  T
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  472 gaactaaaaggaaagattcccgatgtgccaggattctcctgggtg
      E  L  K  G  K  I  P  D  V  P  G  F  S  W  V
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      T  P  C  I  S  A  K  D  I  V  Y  I  G  L  R
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      D  V  D  P  G  E  H  Y  I  L  K  T  L  G  I
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      K  Y  F  S  M  T  E  V  D  R  L  G  I  G  K
  652 gtgatggaagaaacactcagctatctactaggaagaaagaaaagg
      V  M  E  E  T  L  S  Y  L  L  G  R  K  K  R
  697 ccaattcatctaagttttgatgttgacggactggacccatctttc
      P  I  H  L  S  F  D  V  D  G  L  D  P  S  F
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      T  P  A  T  G  T  P  V  V  G  G  L  T  Y  R
  787 gaaggtctctacatcacagaagaaatctacaaaacagggctactc
      E  G  L  Y  I  T  E  E  I  Y  K  T  G  L  L
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      P  E  E  V  T  R  T  V  N  T  A  V  A  I  T
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      L  A  C  F  G  L  A  R  E  G  N  H  K  P  I
  967 gactaccttaacccacctaagtaa 990
      D  Y  L  N  P  P  K  *
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Fig. 3

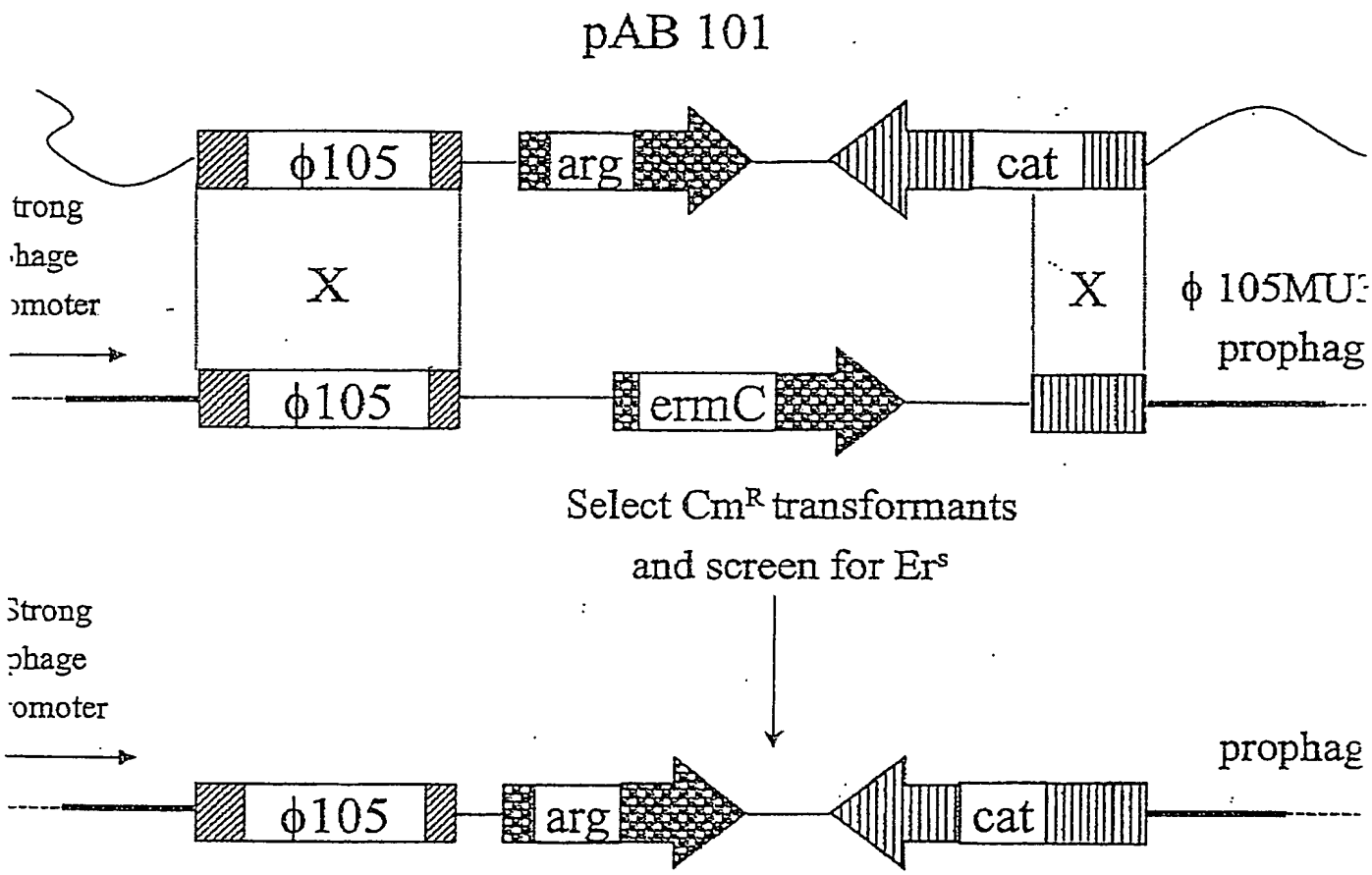
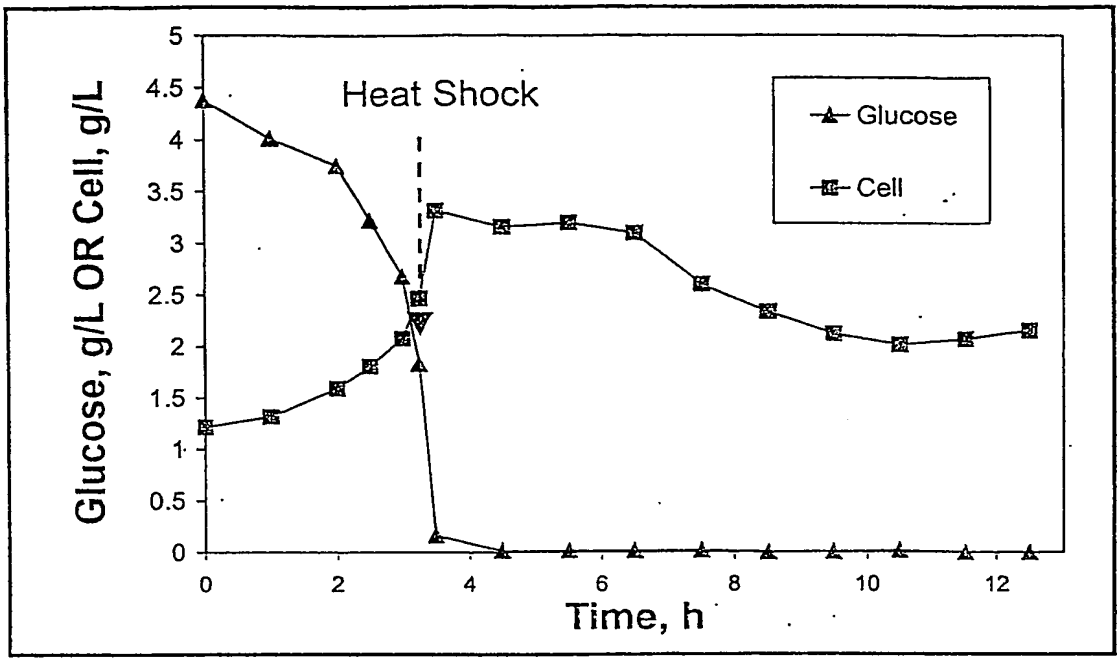

pAB 101
trong
hage
omoter
ϕ105
X
ϕ105
arg
ermC
cat
X
ϕ 105MU
prophag
Select Cm^R transformants
and screen for Er^s
Strong
phage
omoter
ϕ105
arg
cat
prophag

Fig. 4A


Heat Shock
Glucose
Cell
Glucose, g/L OR Cell, g/L
Time, h
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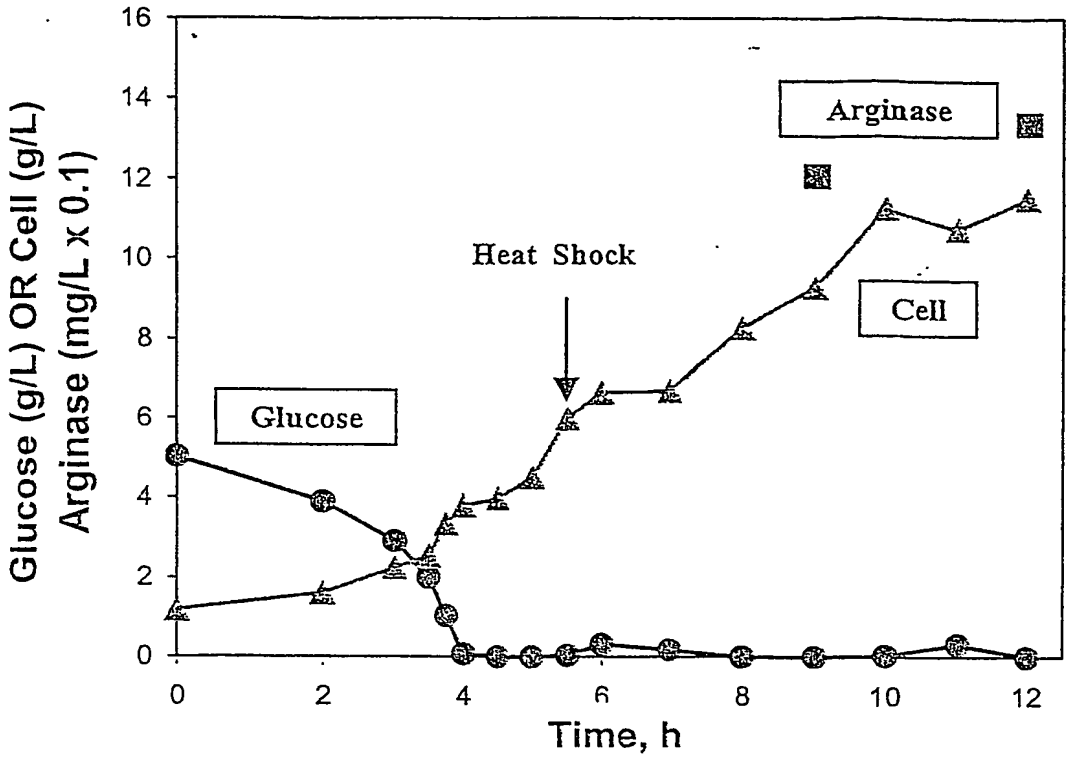
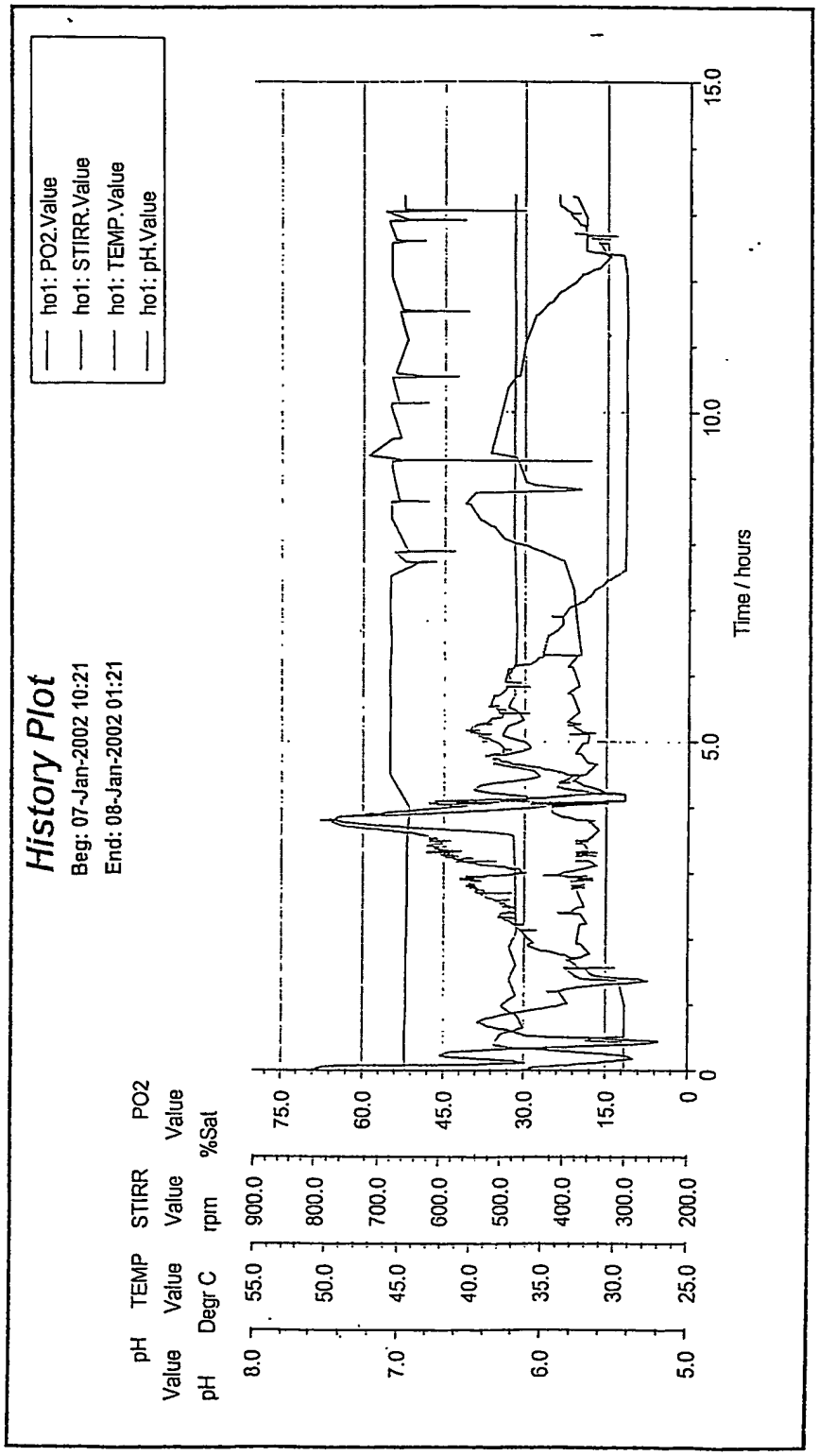
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
0
2
4
6
8
10
12

Fig. 4B


Glucose (g/L) OR Cell (g/L)
Arginase (mg/L x 0.1)
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
12
Time, h
Arginase
Heat Shock
Cell
Glucose


History Plot
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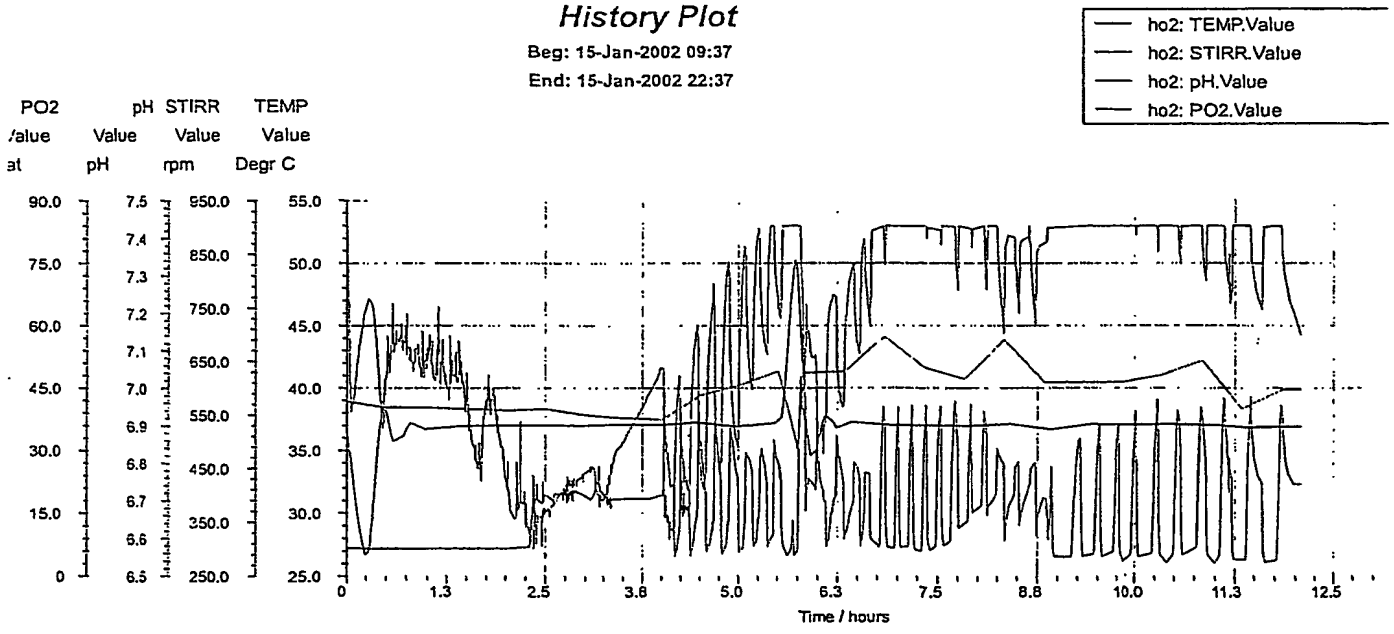
End: 08-Jan-2002 01:21
ho1: PO2.Value
ho1: STIRR.Value
ho1: TEMP.Value
ho1: pH.Value
pH Value
TEMP Value
STIRR Value
PO2 Value
pH
Degr C
rpm
%Sat
Time / hours

Fig. 5A

Fig. 5B


History Plot
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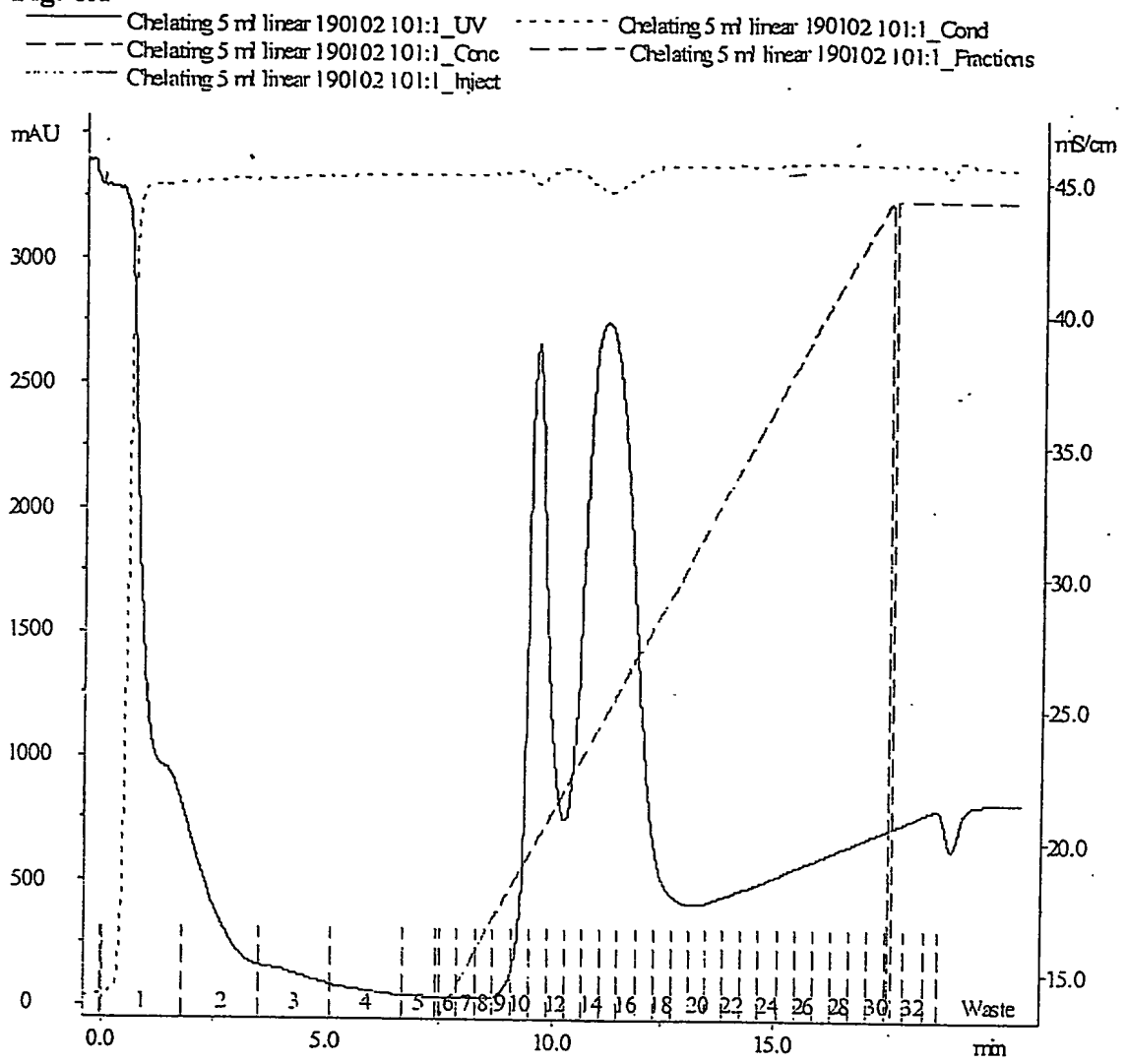
ho2: pH.Value
ho2: PO2.Value
PO2
Value
at
pH
Value
pH
STIRR
Value
rpm
TEMP
Value
Degr C
90.0
75.0
60.0
45.0
30.0
15.0
0
7.5
7.4
7.3
7.2
7.1
7.0
6.9
6.8
6.7
6.6
6.5
950.0
850.0
750.0
650.0
550.0
450.0
350.0
250.0
55.0
50.0
45.0
40.0
35.0
30.0
25.0
0
1.3
2.5
3.8
5.0
6.3
7.5
8.8
10.0
11.3
12.5
Time / hours

Fig. 6A



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Equilibrate_with	0.5 {CV}
Flowthrough_FracSize	8 {ml}
Empty_loop_with	0 {ml}
Wash_column_with	7 {CV}
Start_Frac_at	0 {%B}
Eluate_FracSize	2 {ml}
End_Frac_at	100 {%B}
Target_ConcB_1	100 {%B}
Length_of_gradient_1	10 {base}
Target_ConcB_2	0 {%B}
Length_of_gradient_2	0 {base}
Target_ConcB_3	0 {%B}
Length_of_gradient_3	0.00 {base}
Conc_of_eluent_B	100 {%B}
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Reequilibrate_conc	0.00 {%B}
Reequilibrate_with	0.00 {CV}

Fig. 6B

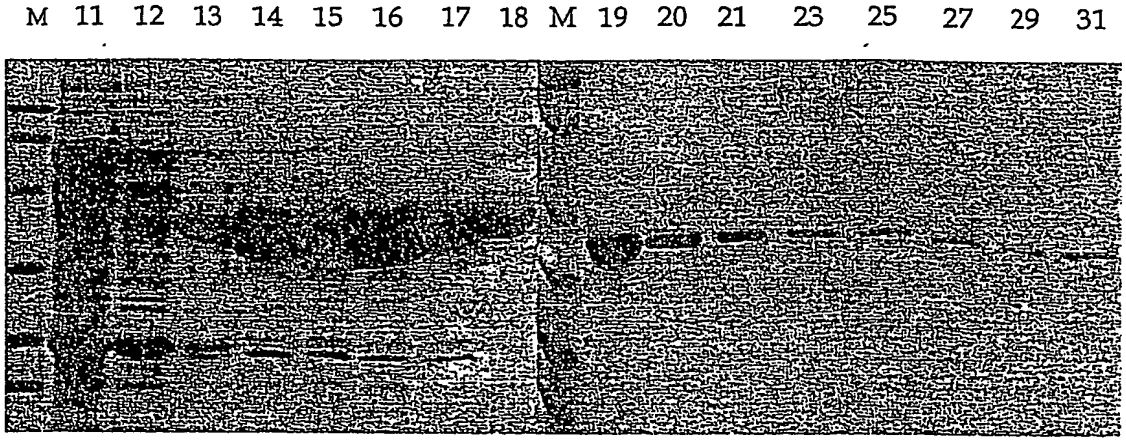
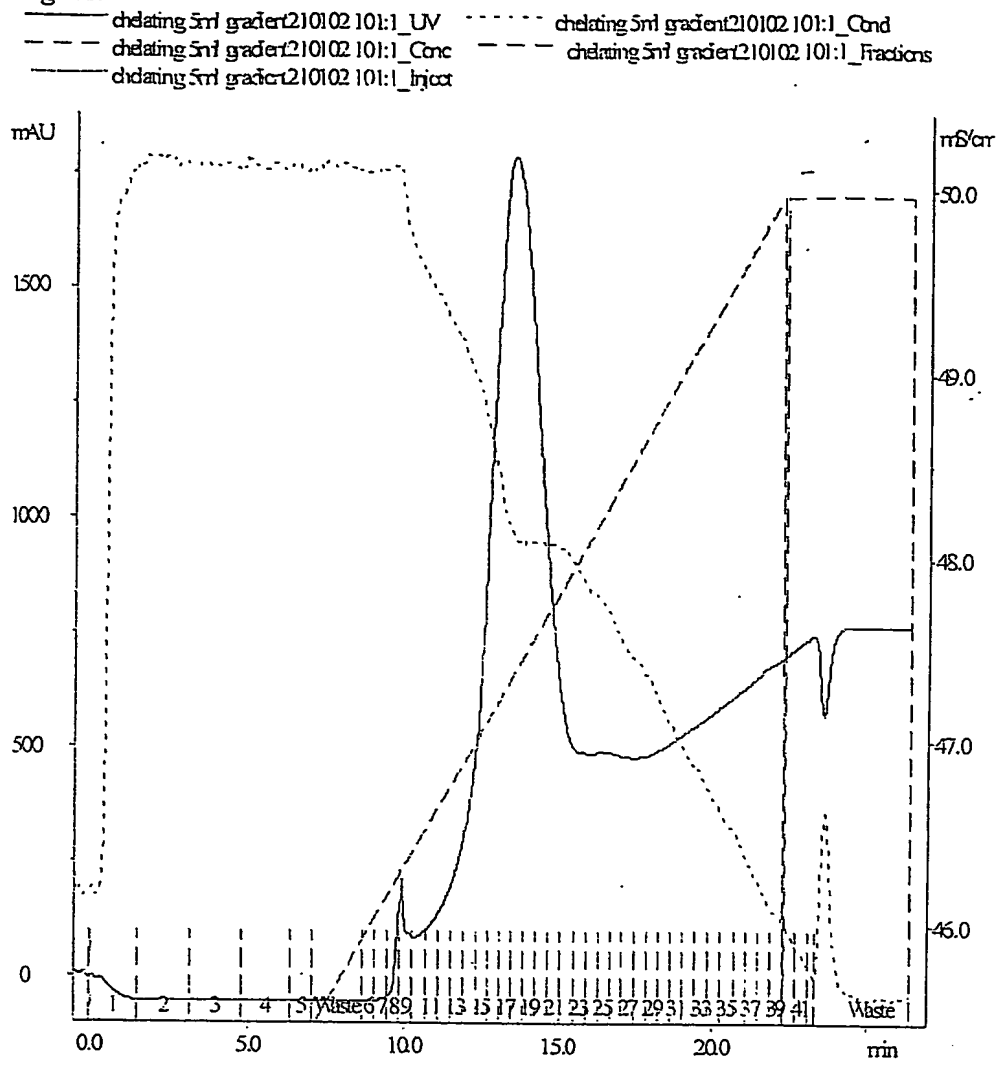


Fig. 7A



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Equilibrate_with	0.5 {CV}
Flowthrough_FracSize	8 {ml}
Empty_loop_with	0 {ml}
Wash_column_with	7 {CV}
Start_Frac_at	10 {%B}
Eluate_FracSize	2 {ml}
End_Frac_at	100 {%B}
Target_ConcB_1	100 {%B}
Length_of_gradient_1	15 {base}
Target_ConcB_2	0 {%B}
Length_of_gradient_2	0.00 {base}
Target_ConcB_3	0 {%B}
Length_of_gradient_3	0.00 {base}
Conc_of_eluent_B	100 {%B}
Clean_with	4.00 {CV}
Reequilibrate_conc	0.00 {%B}
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Fig. 7B

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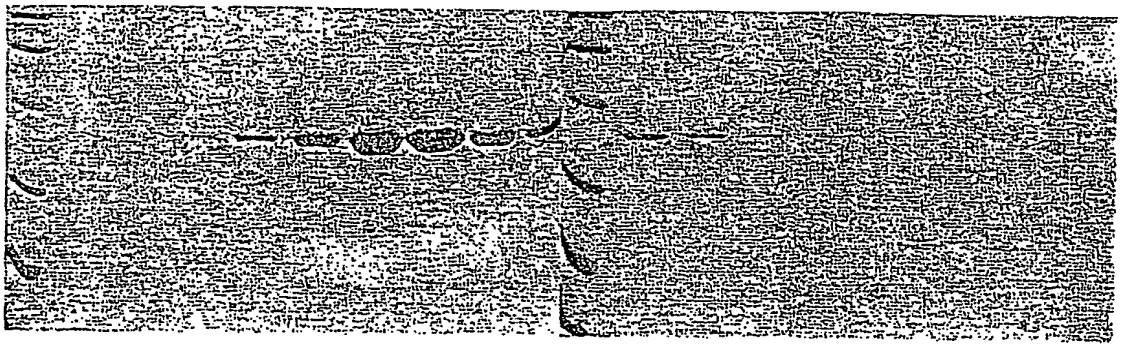
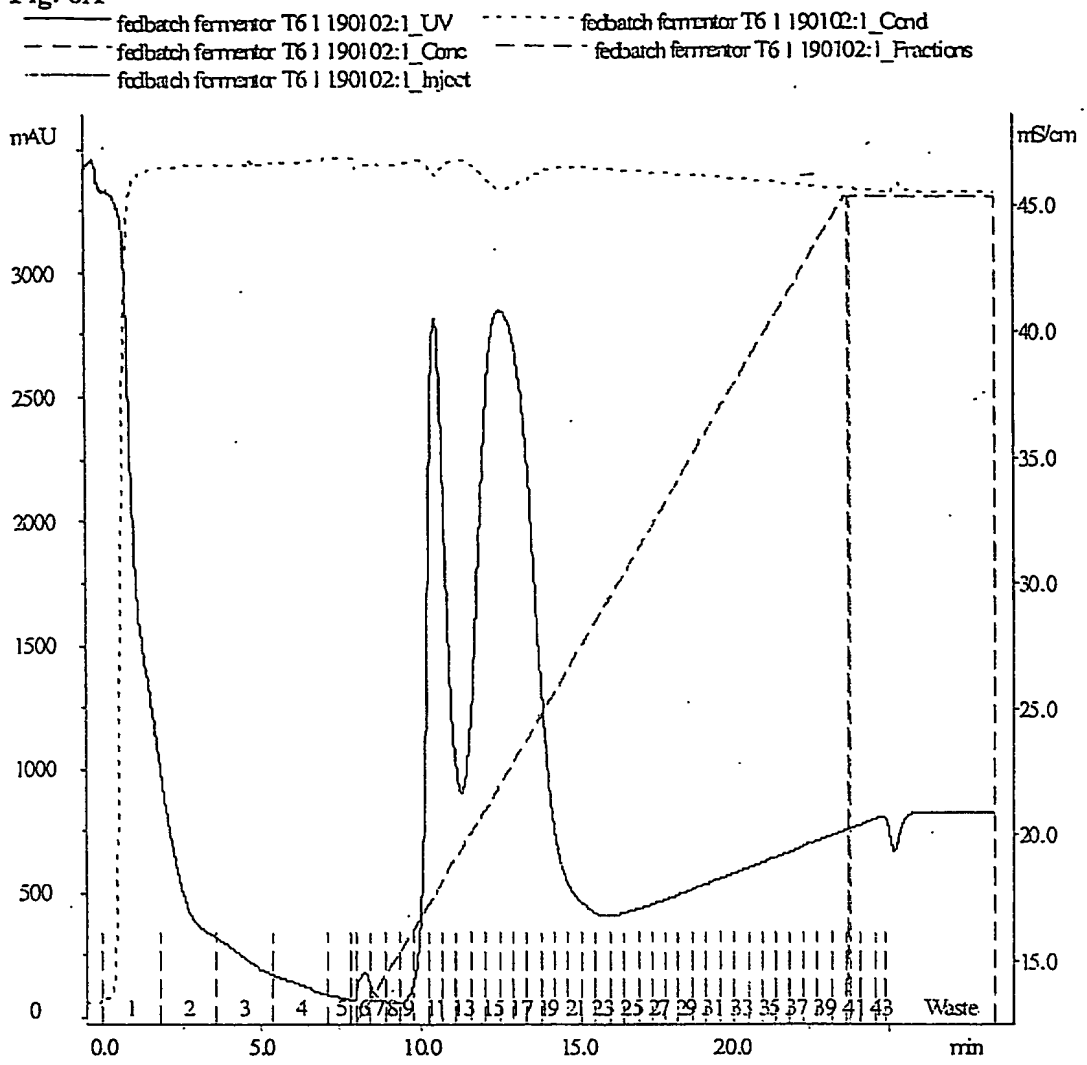


Fig. 8A



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Equilibrate_with	0.5 {CV}
Flowthrough_FracSize	8 {ml}
Empty_loop_with	0 {ml}
Wash_column_with	7 {CV}
Start_Frac_at	0 {%B}
Eluate_FracSize	2 {ml}
End_Frac_at	100 {%B}
Target_ConcB_1	100 {%B}
Length_of_gradient_1	14 {base}
Target_ConcB_2	0 {%B}
Length_of_gradient_2	0.00 {base}
Target_ConcB_3	0 {%B}
Length_of_gradient_3	0.00 {base}
Conc_of_eluent_B	100 {%B}
Clean_with	4.00 {CV}
Reequilibrate_conc	0.00 {%B}
Reequilibrate_with	0.00 {CV}

Fig. 8B

M 10 11 12 13 14 15 16 17 18 M 19 20 21 22 24 26 28 30 32

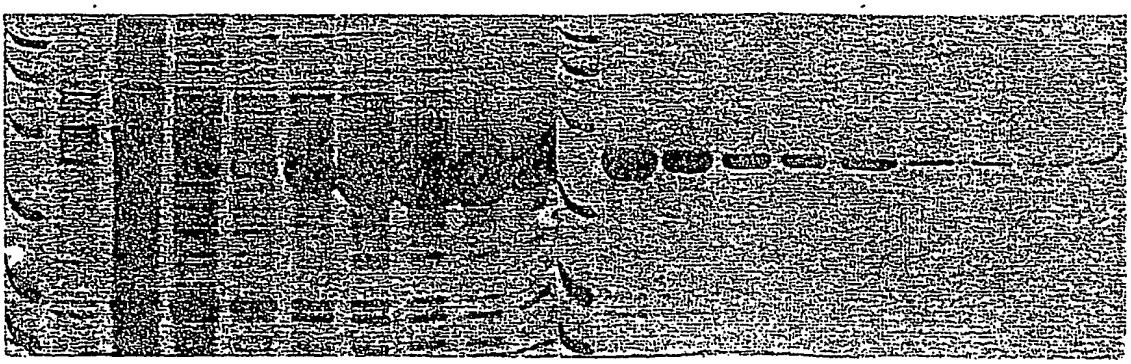
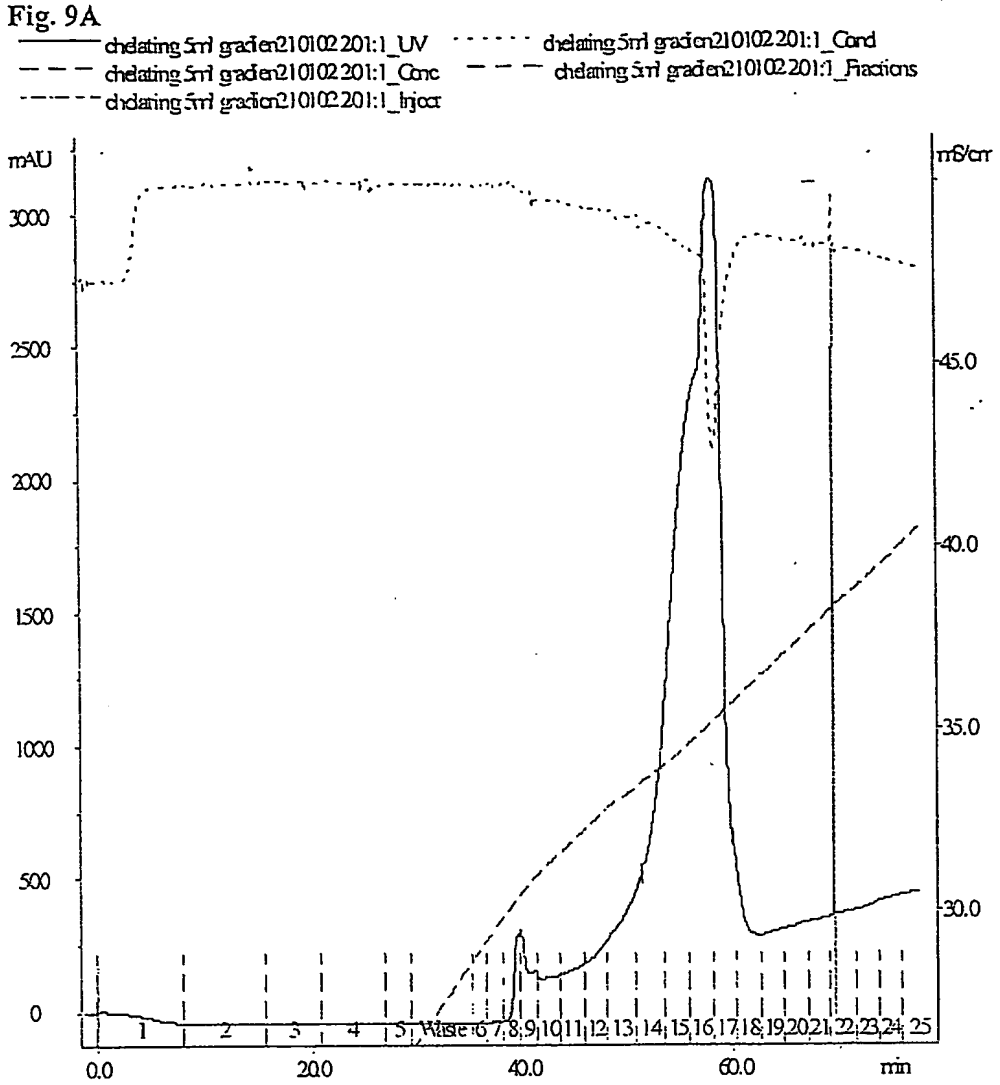


Fig. 9A



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Flow	5.00 {ml/min}
UV_Averaging_time	2.60
Start_ConcB	0.00 {%B}
Equilibrate_with	0.2 {CV}
Flowthrough_FracSize	8 {ml}
Empty_loop_with	0 {ml}
Wash_column_with	7 {CV}
Start_Frac_at	10 {%B}
Eluate_FracSize	2 {ml}
End_Frac_at	100 {%B}
Target_ConcB_1	100 {%B}
Length_of_gradient_1	15 {base}
Target_ConcB_2	0 {%B}
Length_of_gradient_2	0.00 {base}
Target_ConcB_3	0 {%B}
Length_of_gradient_3	0.00 {base}
Conc_of_eluent_B	100 {%B}
Clean_with	4.00 {CV}
Reequilibrate_conc	0.00 {%B}
Reequilibrate_with	0.00 {CV}

Fig. 9B

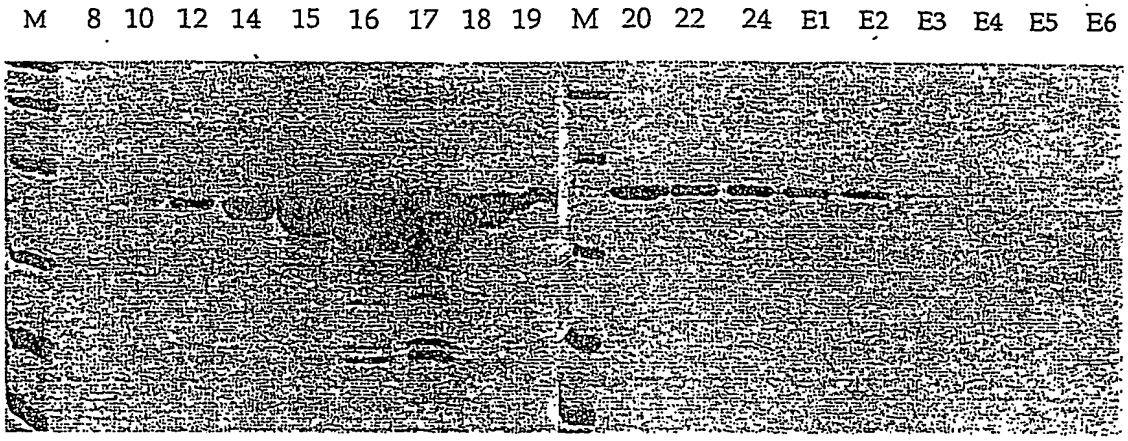


Fig. 10

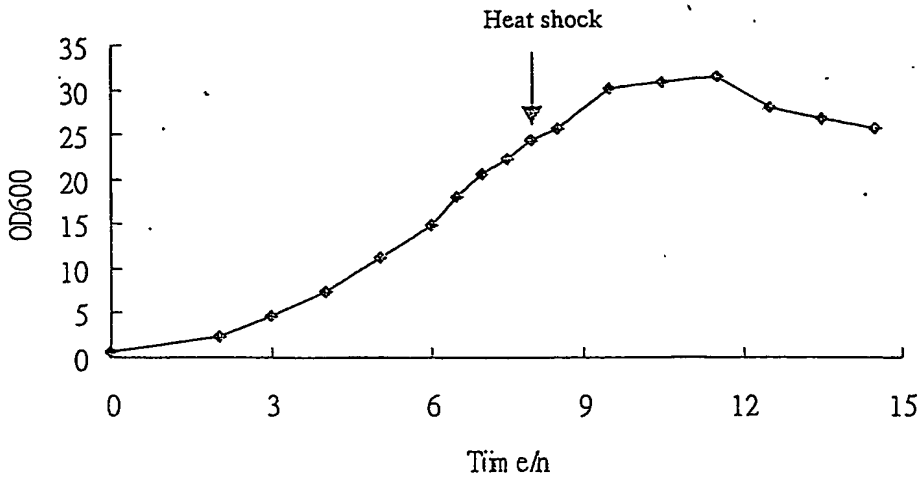


Fig. 11

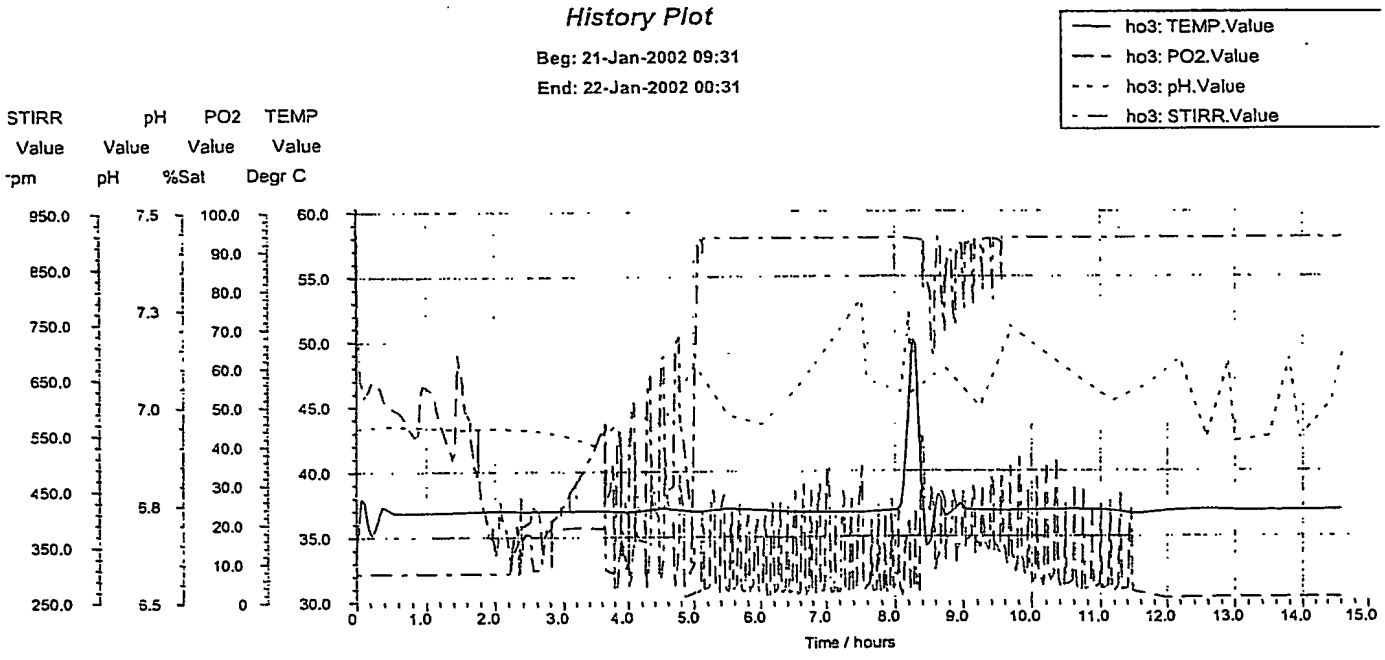
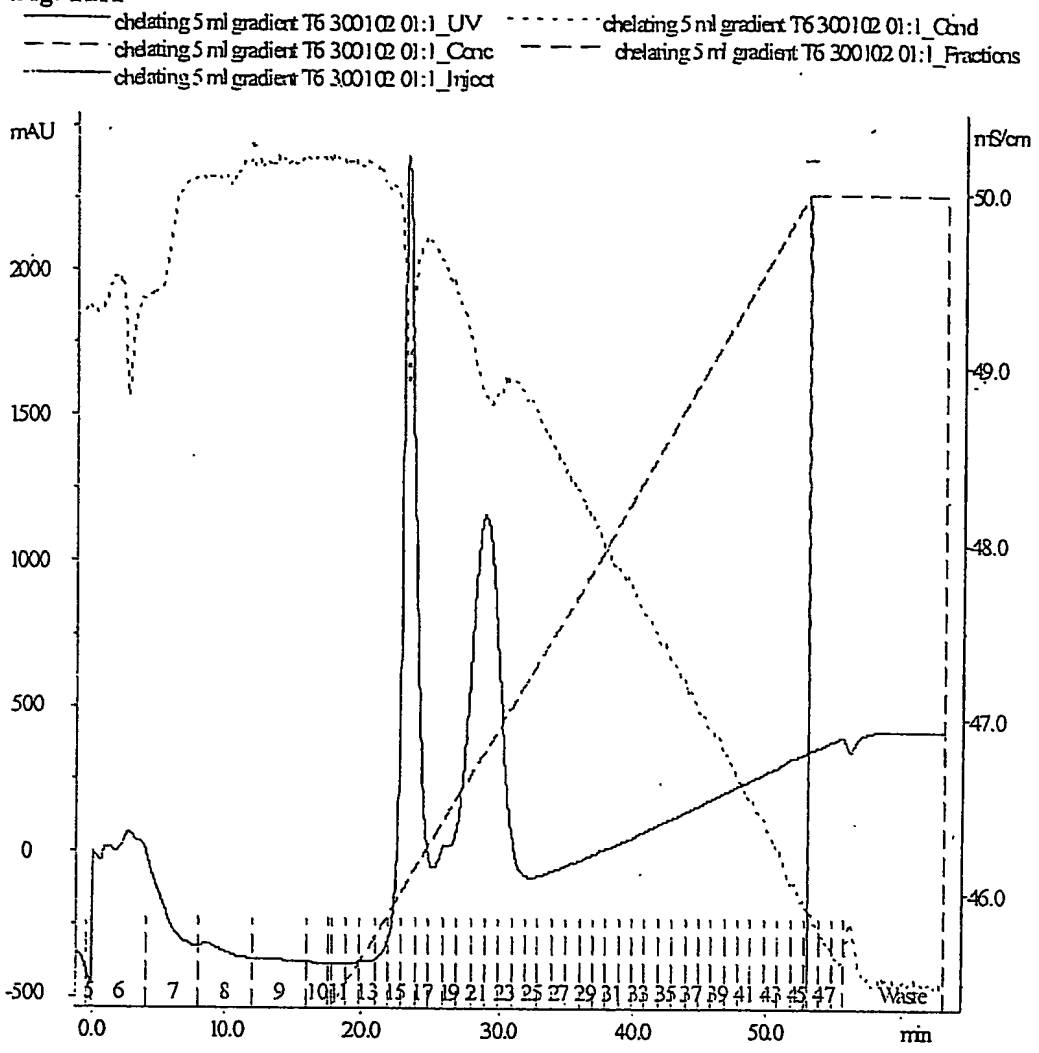

History Plot
Beg: 21-Jan-2002 09:31
End: 22-Jan-2002 00:31
ho3: TEMP.Value
ho3: PO2.Value
ho3: pH.Value
ho3: STIRR.Value
STIRR Value rpm
pH Value pH
PO2 Value %Sat
TEMP Value Degr C
950.0
850.0
750.0
650.0
550.0
450.0
350.0
250.0
7.5
7.3
7.0
6.8
6.5
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0
60.0
55.0
50.0
45.0
40.0
35.0
30.0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0
15.0
Time / hours

Fig. 12A



Column	HiTrap_Chelating_1x5_ml
Pressure_limit	0.5 {MPa}
Flow	2 {ml/min}
UV_Averaging_time	2.60
Start_ConcB	0.00 {%B}
Equilibrate_with	2 {CV}
Flowthrough_FracSize	8 {ml}
Empty_loop_with	0 {ml}
Wash_column_with	7 {CV}
Start_Frac_at	0 {%B}
Eluate_FracSize	2 {ml}
End_Frac_at	100 {%B}
Target_ConcB_1	100 {%B}
Length_of_gradient_1	14 {base}
Target_ConcB_2	0 {%B}
Length_of_gradient_2	0.00 {base}
Target_ConcB_3	0 {%B}
Length_of_gradient_3	0.00 {base}
Conc_of_eluent_B	100 {%B}
Clean_with	4.00 {CV}
Reequilibrate_conc	0.00 {%B}
Reequilibrate_with	0.00 {CV}

Fig. 12B

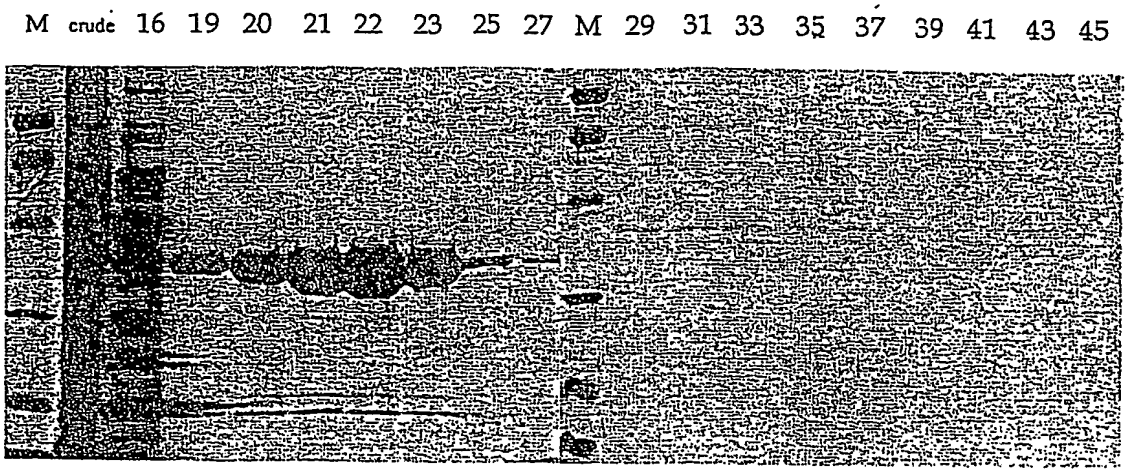
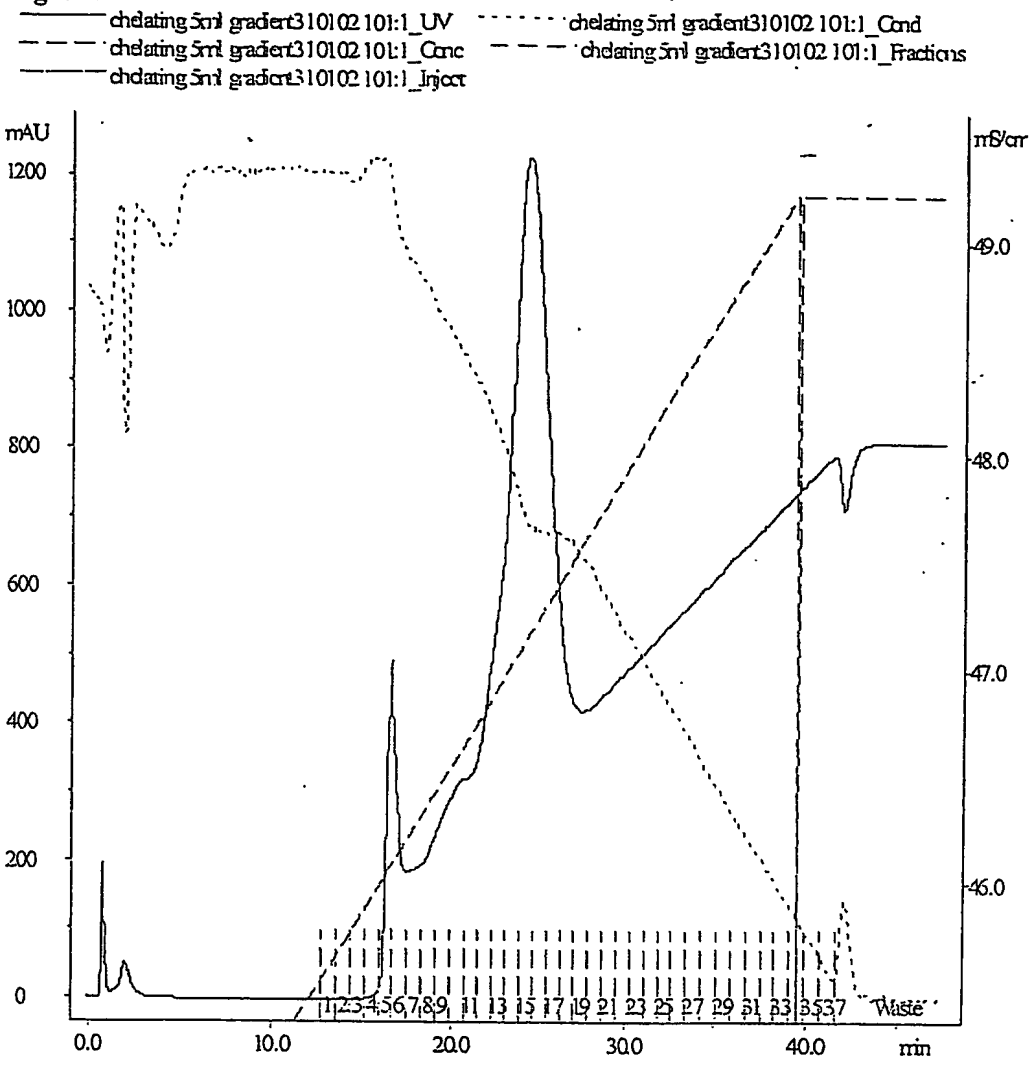


Fig. 13A



Column	HiTrap_Chelating_1x5_ml
Pressure_limit	0.8 {MPa}
Flow	2.5 {ml/min}
UV_Averaging_time	2.60
Start_ConcB	0.00 {%B}
Equilibrate_with	0 {CV}
Flowthrough_FracSize	0 {ml}
Empty_loop_with	0 {ml}
Wash_column_with	5.5 {CV}
Start_Frac_at	5 {%B}
Eluate_FracSize	2 {ml}
End_Frac_at	100 {%B}
Target_ConcB_1	100 {%B}
Length_of_gradient_1	14 {base}
Target_ConcB_2	0 {%B}
Length_of_gradient_2	0.00 {base}
Target_ConcB_3	0 {%B}
Length_of_gradient_3	0.00 {base}
Conc_of_eluent_B	100 {%B}
Clean_with	4.00 {CV}
Reequilibrate_conc	0.00 {%B}
Reequilibrate_with	0.00 {CV}

Fig. 13B

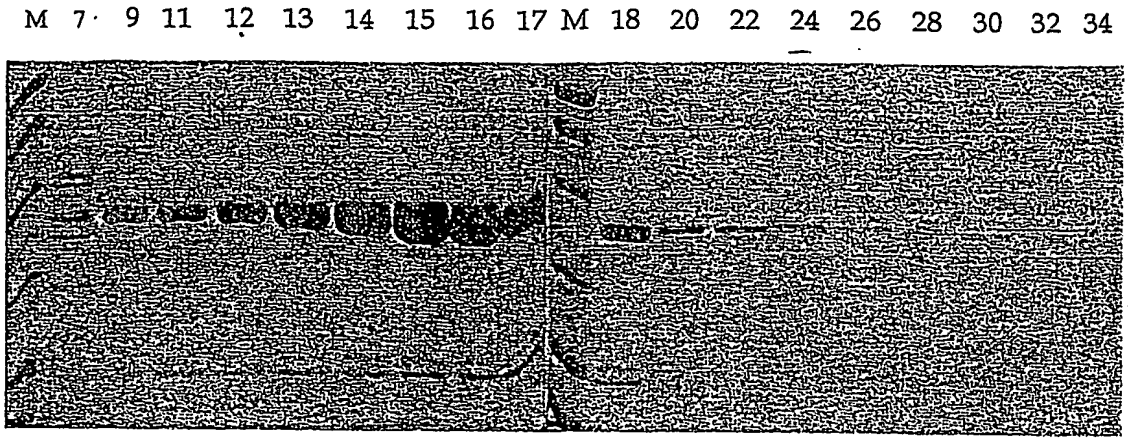
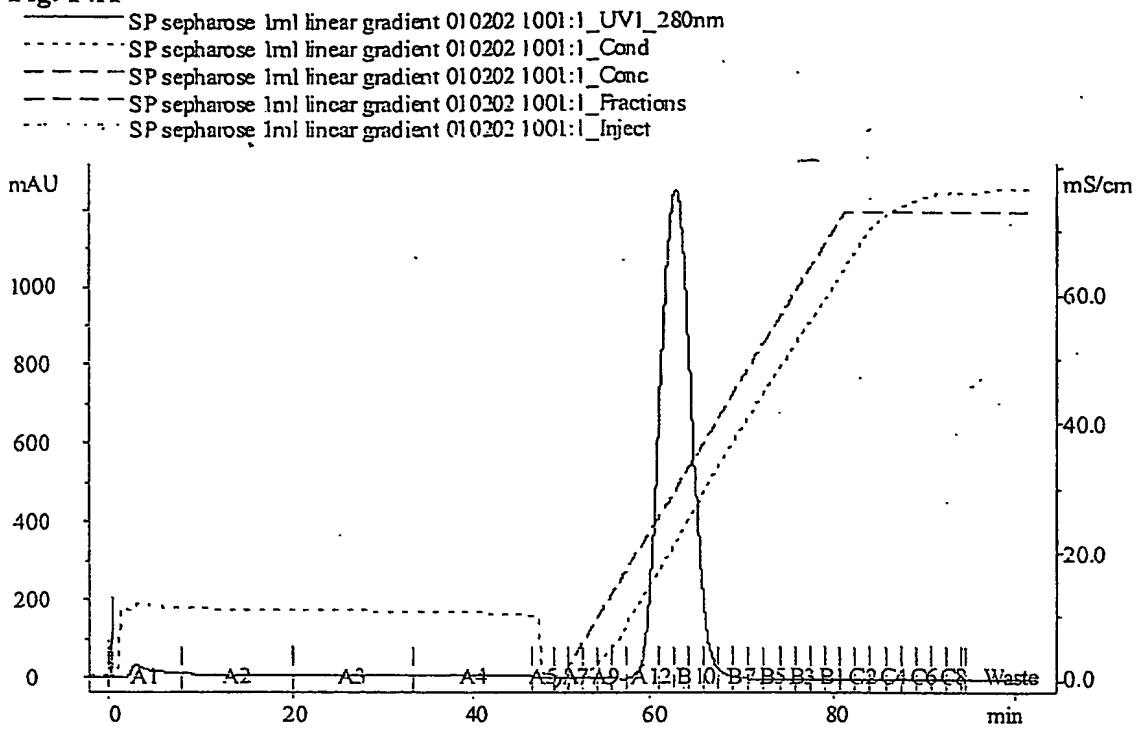


Fig. 14A



Column	HiTrap_SP_FF_1_ml
Flow_Rate	1.00 {ml/min}
Column_PressureLimit	0.40 {MPa}
Wavelength_1	280 {nm}
Wavelength_2	OFF {nm}
Wavelength_3	OFF {nm}
Averaging_Time_UV	5.12 {sec}
Pump_A_Inlet	A1
Pump_B_Inlet	B1
Wash_Inlet_A1_	OFF
Wash_Inlet_A2_	OFF
Wash_Inlet_B1_	OFF
Wash_Inlet_B2_	OFF
Start_ConcB	0 {%B}
Compensation_Volume	8 {ml}
Equilibrate_with	0 {CV}
Flowthrough_TubeType	18mm
Flowthrough_FracSize	8 {ml}
Flowthrough_StartAt	FirstTube
Empty_loop_with	31.500 {ml}
Wash_column_with	2 {CV}
_Start_Frac_at	0 {%B}
_End_Frac_at	100 {%B}
TubeType_EluateFrac	18mm
Eluate_Frac_Size	1 {ml}
EluateFrac_StartAt	NextTube
Target_ConcB	100 {%B}
Length_of_Gradient	20.00 {base}
Gradient_Delay	8 {ml}
Clean_with	5.00 {CV}

Fig. 14B

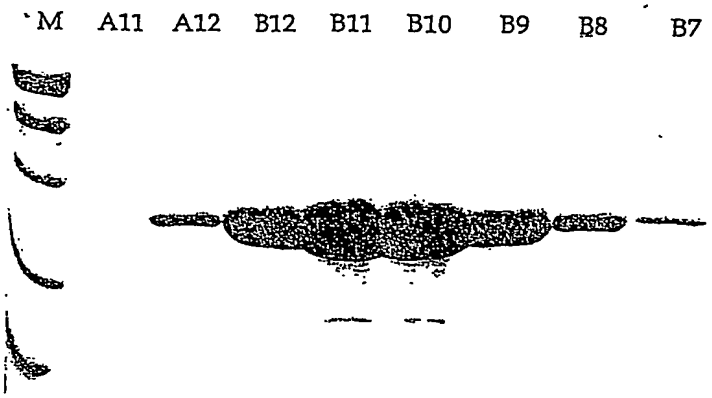
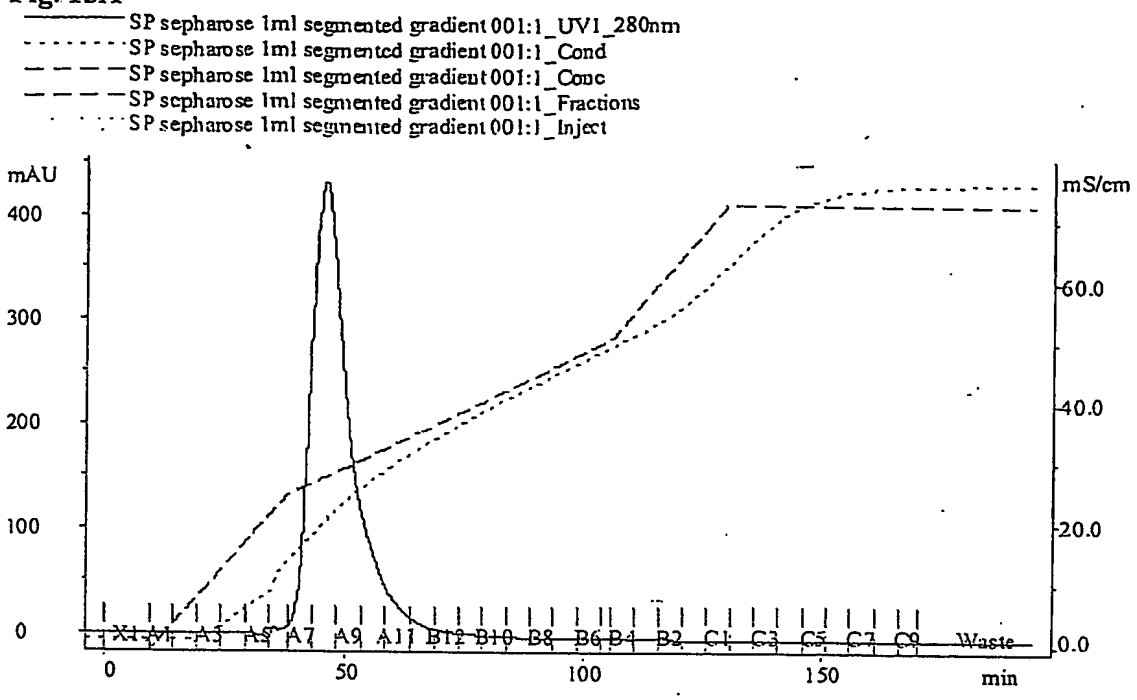


Fig. 15A



Column HiTrap_SP_FF_1_ml
Flow_Rate 0.40 {ml/min}
Column_PressureLimit 0.50 {MPa}
Wavelength_1 280 {nm}
Wavelength_2 OFF {nm}
Wavelength_3 OFF {nm}
Averaging_Time_UV 5.12 {sec}
Pump_A_Inlet A1
Pump_B_Inlet B1
Wash_Inlet_A1_ OFF
Wash_Inlet_A2_ OFF
Wash_Inlet_B1_ OFF
Wash_Inlet_B2_ OFF
Start_ConcB 0 {%B}
Compensation_Volume 8 {ml}
Equilibrate_with 0 {CV}
Flowthrough_TubeType 30mm
Flowthrough_FracSize 40 {ml}
Flowthrough_StartAt TubeNumber[X.1]
Empty_loop_with 0.000 {ml}
Wash_column_with 2 {CV}
1_Tube_Type 18mm
1_Fraction_Size 1 {ml}
1_Start_at FirstTube
Target_ConcB_1 35 {%B}
Length_of_Gradient_1 6.00 {base}
2_Tube_Type 18mm
2_Fraction_Size 1 {ml}
2_Start_at NextTube
Target_ConcB_2 70 {%B}
Length_of_Gradient_2 14.00 {base}
3_Tube_Type 18mm
3_Fraction_Size 1 {ml}
3_Start_at NextTube
Target_ConcB_3 100 {%B}
Length_of_Gradient_3 5.00 {base}
Gradient_Delay 8 {ml}
Clean_with 5.00 {CV}

Fig. 15B

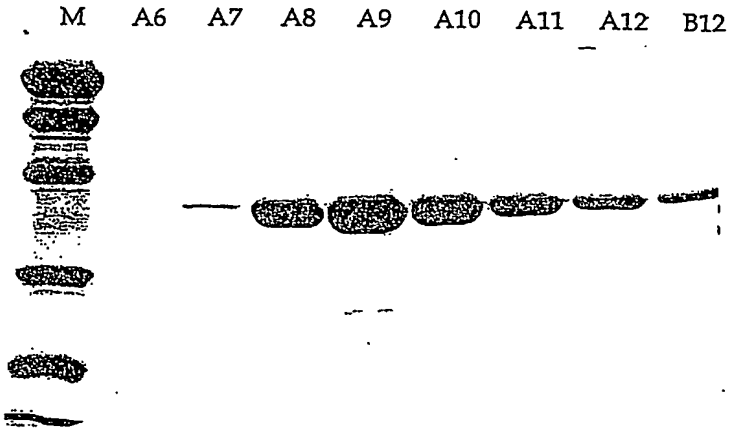


Fig. 16A

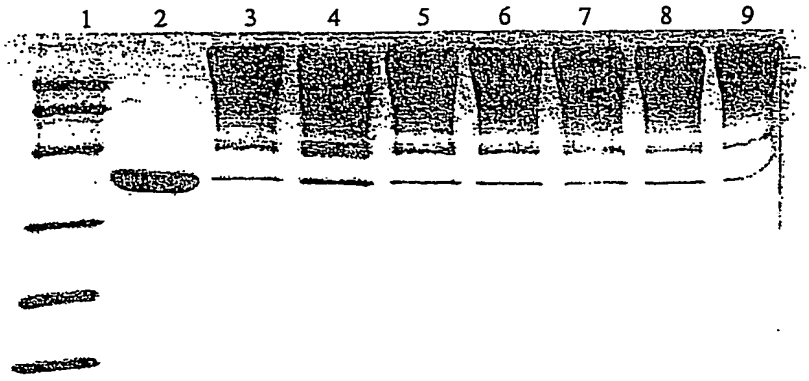


Fig. 16B

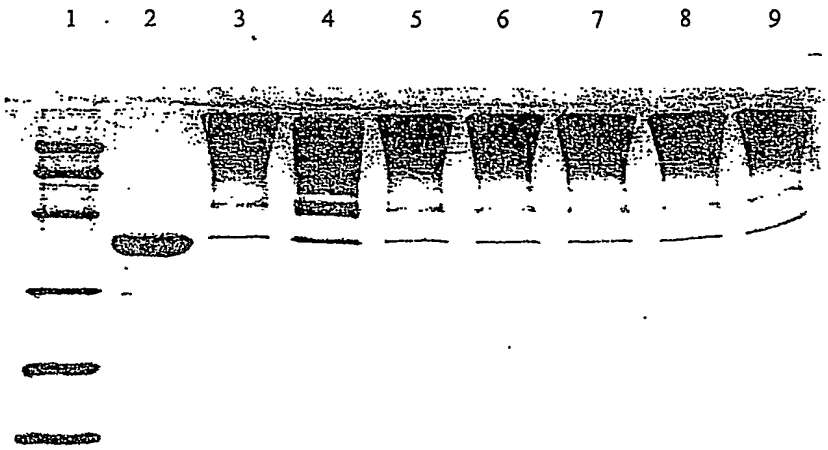


Fig. 17A

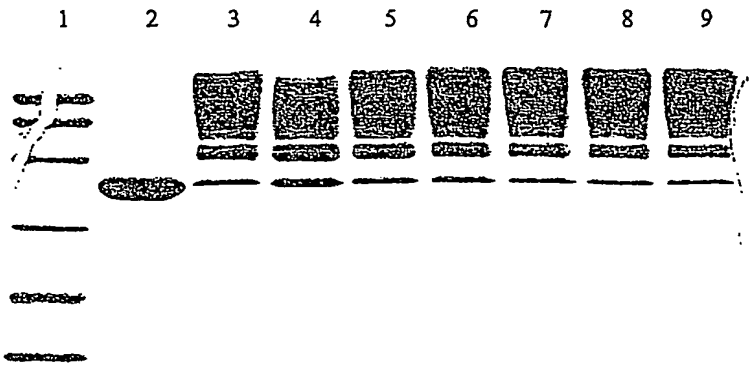


Fig. 17B

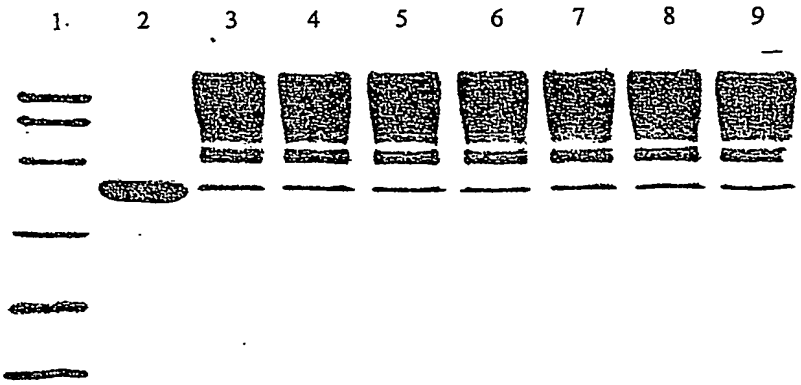


Fig. 18

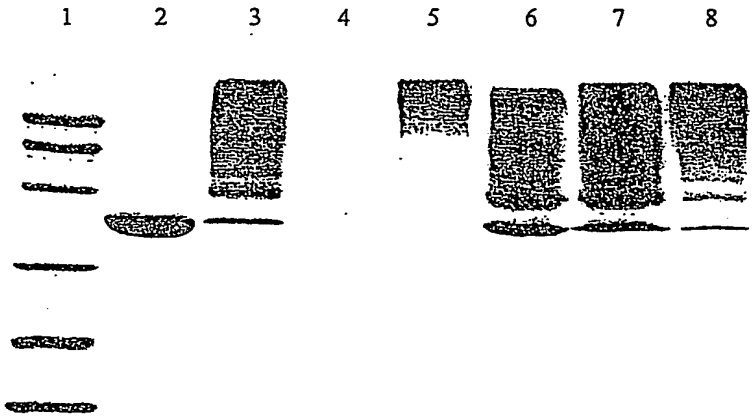


Fig. 19A

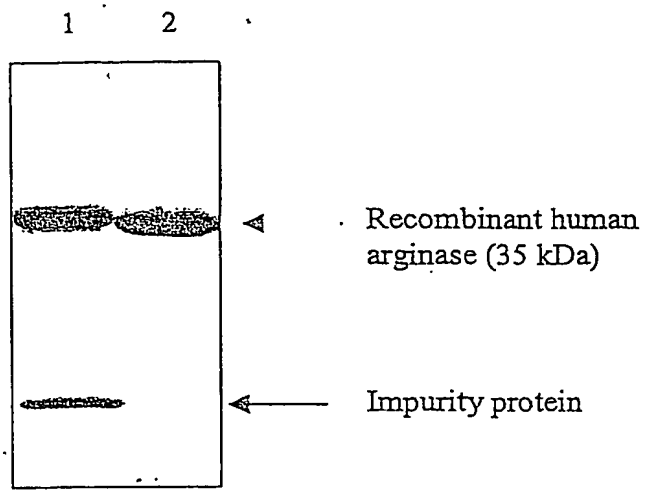


Fig. 19B

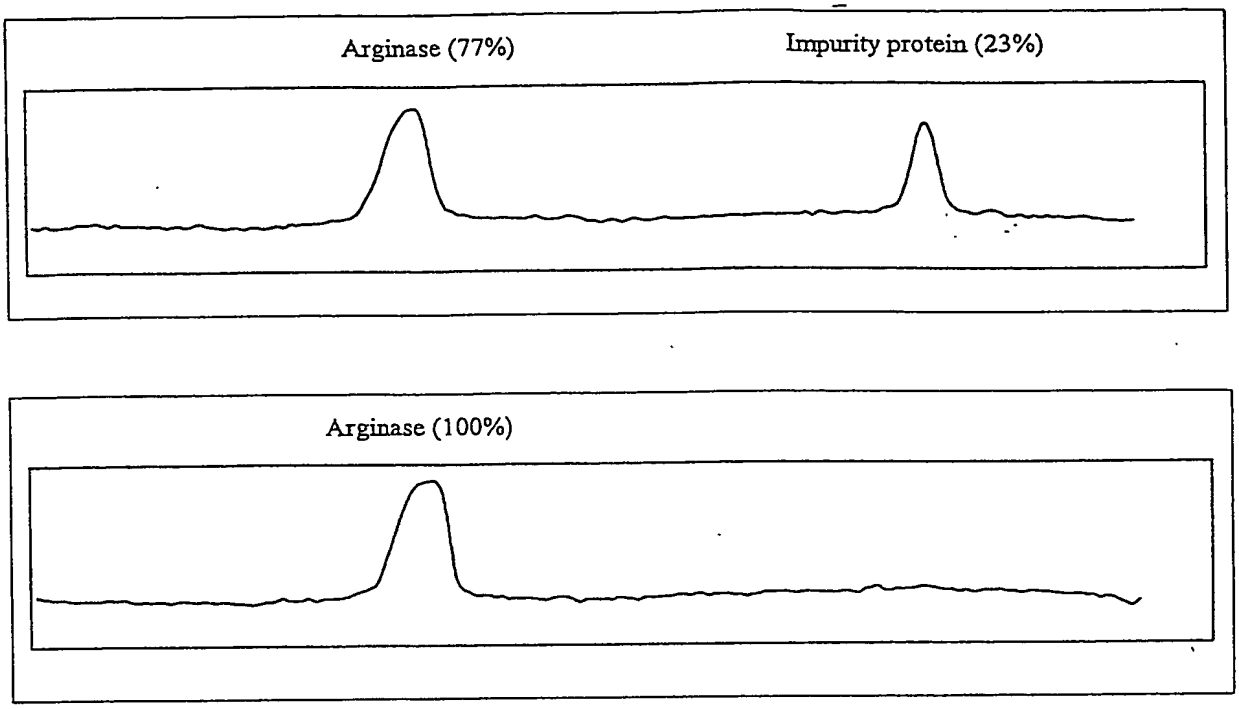


Fig. 20

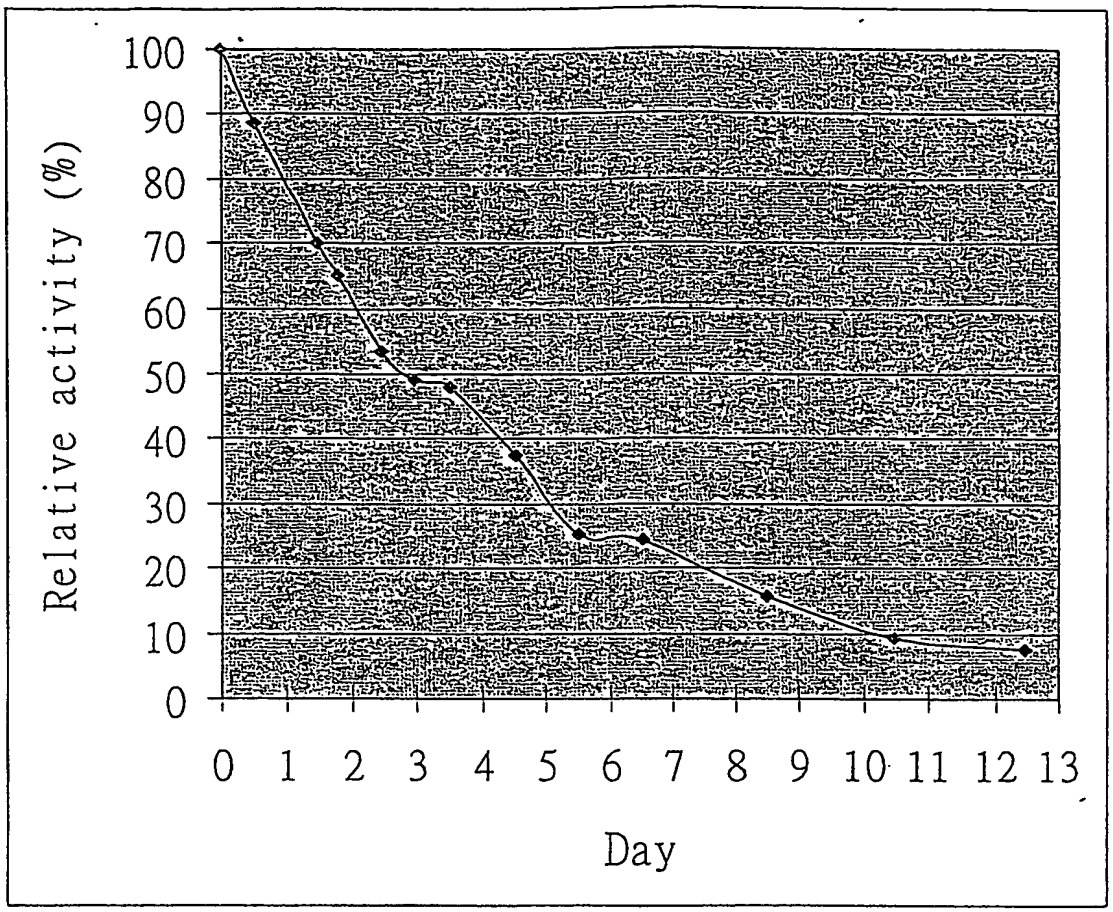
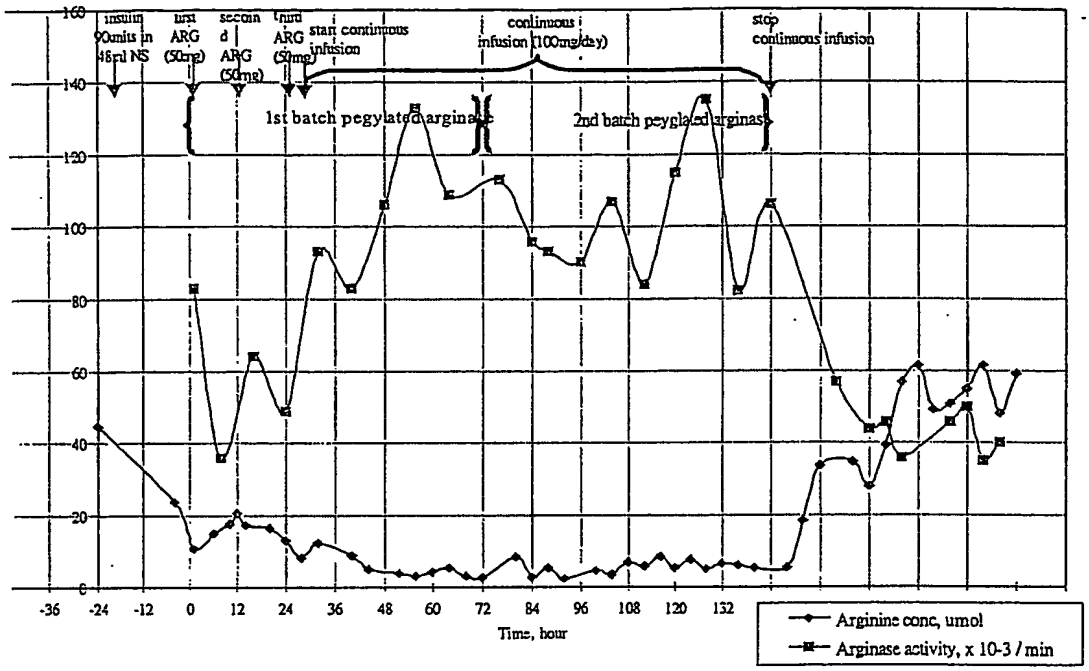
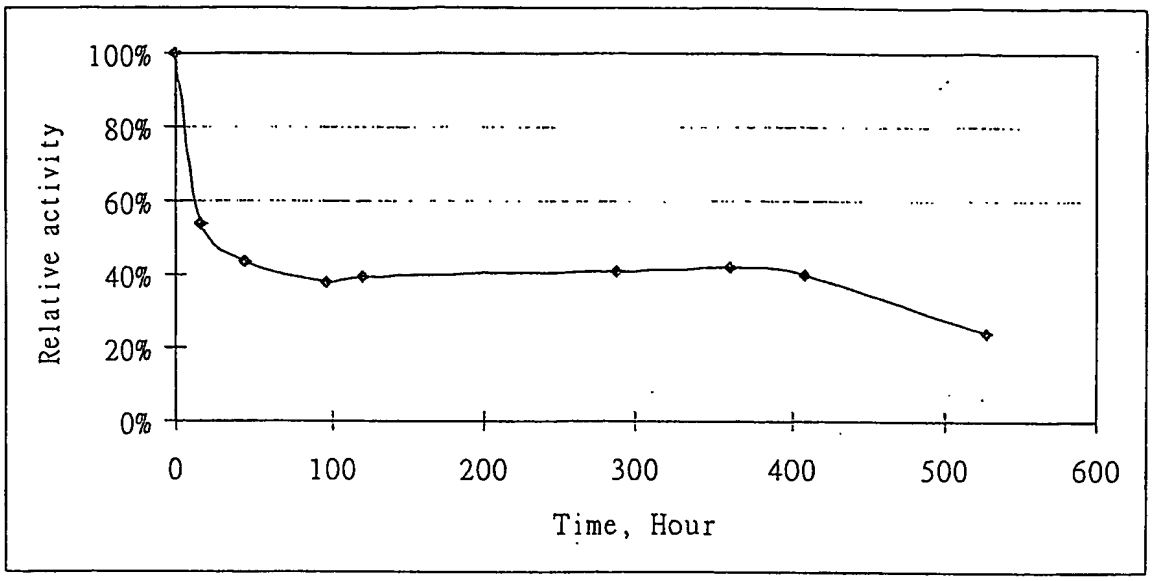

Relative activity (%)
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Day

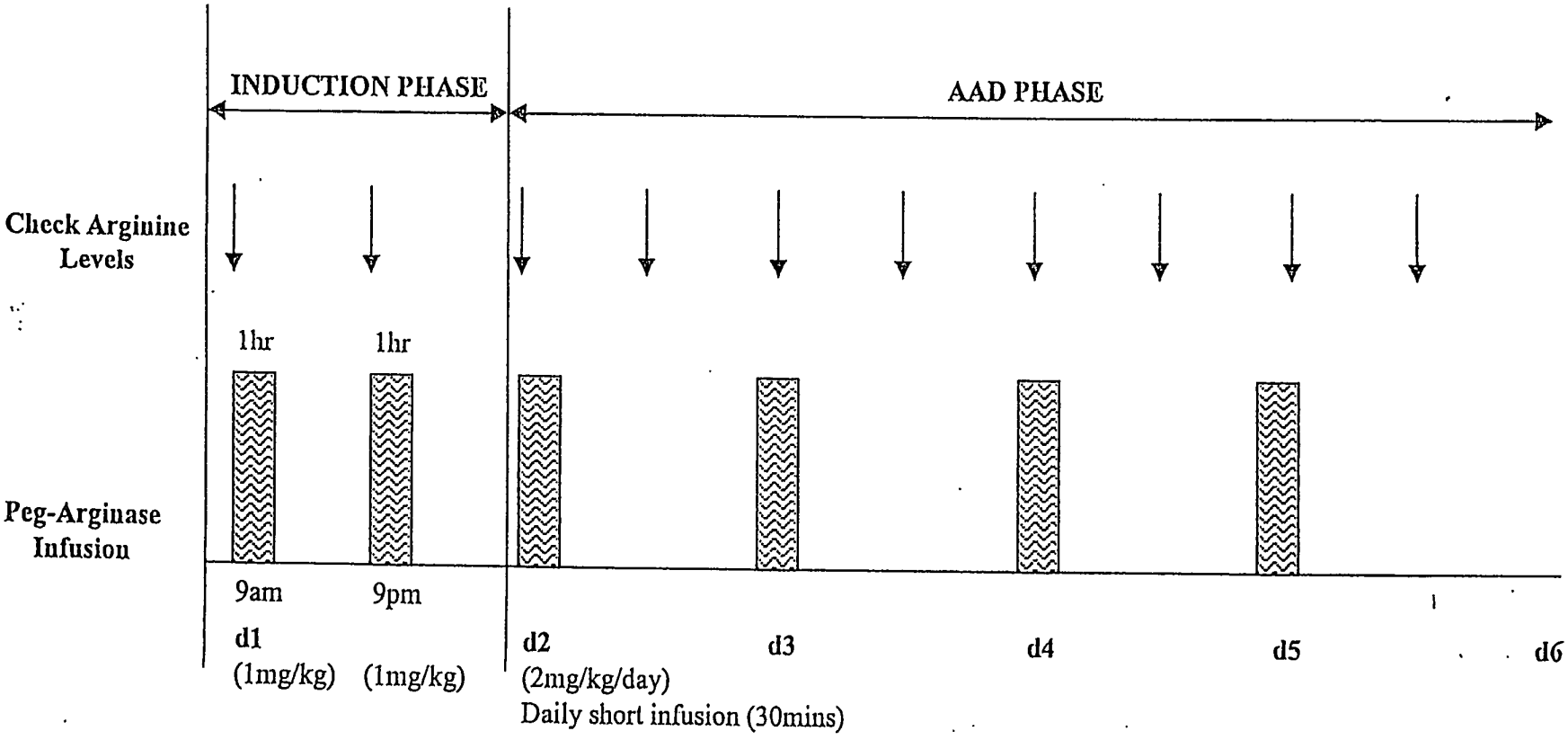
Fig. 21A


insulin 90units in 46ml NS
first ARG (50mg)
second ARG (50mg)
third ARG (50mg)
start continuous infusion
continuous infusion (100mg/day)
stop continuous infusion
1st batch pegylated arginase
2nd batch pegylated arginase
160
140
120
100
80
60
40
20
0
-36
-24
-12
0
12
24
36
48
60
72
84
96
108
120
132
Time, hour
Arginine conc, umol
Arginase activity, x 10-3 / min


100%
80%
60%
40%
20%
0%
Relative activity
0
100
200
300
400
500
600
Time, Hour

TREATMENT SCHEMA

Fig. 22A

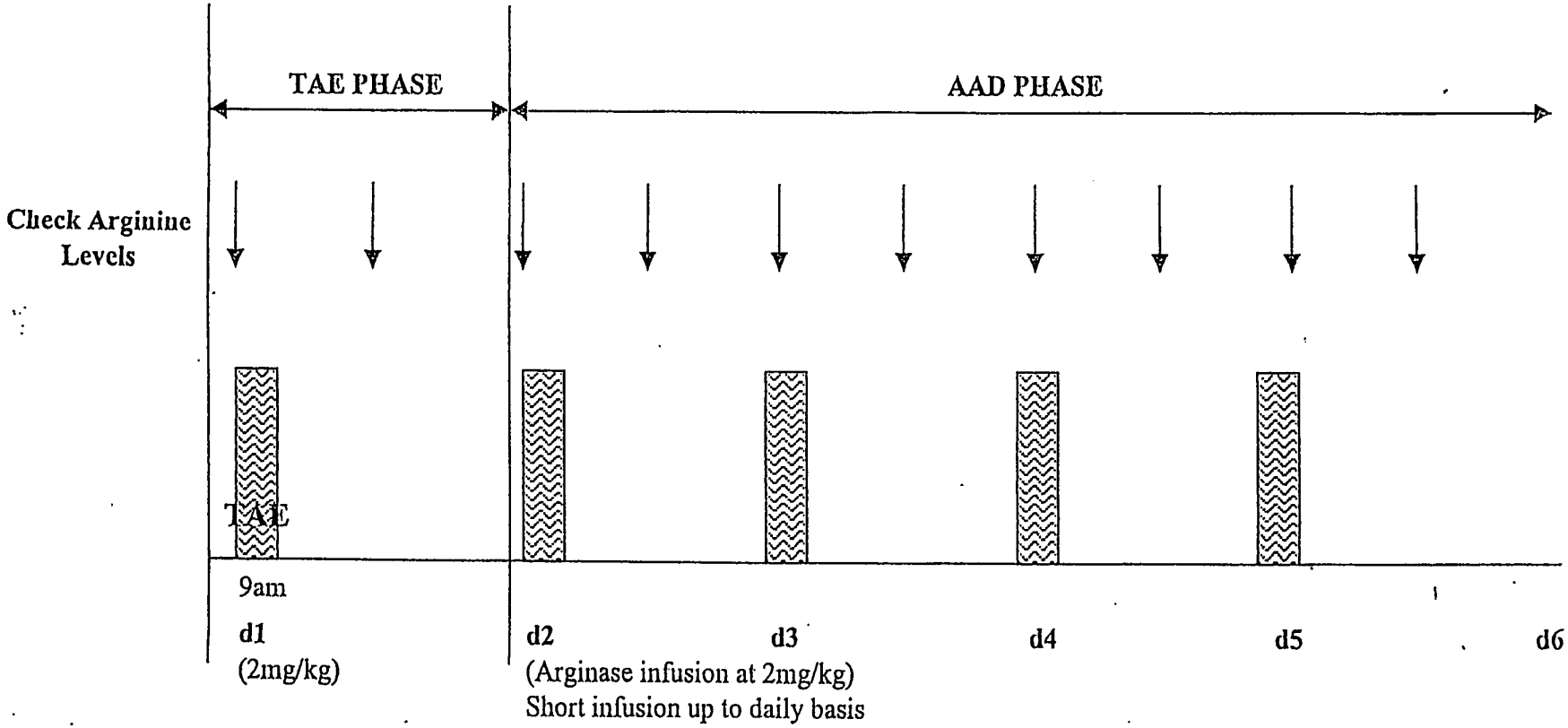


Legends: -

AAD = Adequate Arginine Deprivation

TREATMENT SCHEMA

Fig. 23



Legends: -

AAD = Adequate Arginine Deprivation

TAE = Transphepatic Arterial Embolisation

SEQUENCE LISTING

THE FOLLOWING SEQUENCES ARE DISCLOSED IN THE SPECIFICATION AND NEED TO BE INCLUDED IN A SEQUENCE LISTING

SEQ ID NO: 1 (Fig. 2A)

SEQ ID NO: 2 & 3 (Fig. 2B: nucleotide sequence (SEQ ID NO:2); and amino acid sequence (SEQ ID NO:3))

SEQ ID NO: 4: 6xHis tag MHHHHH

SEQ ID NO: 5: 5' -CCAAACCATATGAGCGCCAAGTCCAGAACCATA-3' (Arg 1)

SEQ ID NO: 6: 5' -CCAAACTCTAGAATCACATTTTTTGAATGACATGGACAC-3' (Arg 2)

SEQ ID NO: 7:: 5' -CTCTGGCCATGCCAGGGTCCACCC-3' (Arg 6)

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